University of Alberta

Lipid Rafts Mediate β1 Integrin Endocytosis in Monocytes

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Medical Sciences - Oncology

Edmonton, Alberta
Spring 2005

0-494-08123-6

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ABSTRACT

Endocytosis is the mechanism by which proteins on the plasma membrane are sorted into vesicles for the purpose of intracellular trafficking. The primary machinery for endocytosis involves protein sorting via clathrin coats. However, recently glycolipid-enriched membrane microdomains or "lipid rafts" are emerging as a lipid-sorting based alternative to clathrin-mediated endocytosis. Here we demonstrate for the first time that clustering $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins on monocytes results in rapid internalization via a cholesterol- and lipid raft-dependent mechanism. Cross-linking fibronectin also promotes cholesterol-dependent internalization. Since clustering the raft marker GM1 was sufficient to induce endocytosis we conclude that monocyte rafts contain machinery for endocytosis which can be activated by lipid aggregation. Lipid raft-mediated endocytosis requires the activation of a serine/threonine kinase and cytoskeletal re-organization. We show that tetraspanin CD81 expression affects the amount of $\beta 1$ integrin endocytosis indicating CD81 is a specific modulator of raft-based endocytosis.

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LIST OF ABBREVIATIONS

Ab	Antibody
AML	Acute myelogenous leukemia
BCR	B-cell receptor
BSA	Bovine serum albumin
CSB	Cytoskeletal stabilizing buffer
CytoD	Cytochalasin D
CtxB	Cholera toxin B-subunit
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GPI	Glycosylphosphatidylinositol
GTP	Guanine triphosphate
ICAM-1	Intracellular adhesion molecule-1
Ig	Immunoglobulin
mAb	Monoclonal antibody
MβCD	Methyl-β-cyclodextrin
MHC	Major histocompatibility complex
Noc	Nocodazole
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI-3 kinase	Phosphatidylinositol 3-kinase
PKC	Protein kinase C

PFA	Paraformaldehyde
RT PCR	Reverse transcription polymerase chain reaction

Chapter 1: Introduction

Section 1.1 Overview and justification of the hypothesis

Endocytosis is the process by which proteins at the cell surface are sorted into vesicles for transport to intracellular locations. The primary machinery for sorting involves association of proteins with clathrin coats [1, 2]. This is mediated by tetrameric adaptor proteins which recognize sorting signals in the cytoplasmic tails of integral membrane proteins and link those proteins to clathrin. Oligomerization of clathrin leads to changes in membrane curvature and formation of clathrin-coated pits from which endocytic vesicles bud [3]. Recently, it has become recognized that lipid-based sorting mechanisms provide an alternative to protein-based mechanisms [2, 4, 5]. Lipid rafts are membrane microdomains formed through the spontaneous aggregation of membrane glycolipids and cholesterol into assemblies of distinct phase and biological properties [4]. Lipid rafts act as sorting devices, admitting proteins with an affinity for their lipid environment, while excluding the majority of transmembrane proteins [6]. By accumulating lipid-modified signaling molecules, and admitting or excluding transmembrane receptor proteins, lipid rafts are believed to regulate the generation of signals at the plasma membrane. Interactions between multivalent ligands and receptors, which increase the affinity of receptors for lipid rafts, result in the combinatorial assembly of signaling molecules within a common platform and the generation of compartmentalized signals [7]. Those

signals regulate a variety of events including changes in gene expression, cytoskeletal reorganization and membrane trafficking [8].

Recognition that a wide variety of viruses, toxins and bacteria gain entry to cells by binding to and aggregating glycolipids was the first indication that lipid rafts could mediate endocytosis [9, 10]. Similarly, clustering of glycosylphosphatidylinositol (GPI)-anchored proteins, which preferentially sort into lipid rafts, leads to their incorporation into endocytic vesicles [11]. Thus lipid-based sorting mechanisms involving lipid rafts provide an effective alternative to clathrin-mediated endocytosis. Until recently there was little evidence that membrane proteins could endocytose using lipid sorting mechanisms. However, it is now established that the glucagon-like peptide-2 (GLP-2) receptor [12], carboxypeptidase E [13] and antigen-bound B-cell receptor internalize through the induction of lipid raft mediated endocytosis [14].

β1 integrins are adhesion receptors that anchor cells to the extracellular matrix (ECM) through re-organization of the actin cytoskeleton under the regulation of the small GTP-binding proteins Rac and Rho [15]. Upon cross-linking with secondary antibodies many integrins undergo rapid internalization in a variety of cell lines [16-18]. However, in most cases the involvement of lipid rafts has not been investigated. α4β1 integrins require interaction with lipid rafts to actively bind ligand [19]. It was recently demonstrated that β1 integrin engagement by the ECM recruits Rac to lipid rafts in adherent cells thereby regulating cytoskeletal reorganization. Importantly, if cells are detached from ECM, lipid rafts rapidly endocytose returning Rac to the cell interior [8].

Therefore, raft-mediated endocytosis is a mechanism that can return cells to a non-adhesive state. The reciprocal and interdependent nature of β1 integrin lipid raft interactions prompted us to investigate whether clustering β1 integrins through interaction with soluble multivalent ligands could activate lipid raft mediated endocytosis.

β1 integrins trigger different effector pathways in different cell types. Therefore, not all cells may internalize integrins via a lipid raft pathway. We chose to investigate cells of the monocytic lineage for the following reasons: 1) we had recently conducted a proteomic survey of monocyte lipid raft proteins that identified proteins involved in membrane trafficking [20, 21] 2) monocytes adhere to the ECM protein fibronectin through engagement of both α4β1 and α5β1 integrins providing an opportunity to investigate whether these integrins differentially regulate interactions with lipid rafts [22, 23] and 3) monocytes extravasate through rapid changes in the formation and dissolution of attachments to vessel wall components which could necessitate cytoskeletal reorganization within lipid rafts [24].

How integrins associate with lipid rafts has not been elucidated. Tetraspanins are a family of multiply inserted transmembrane proteins which are believed to reside within a subset of lipid rafts, activate downstream signals, and interact with numerous membrane proteins, including integrins and other tetraspanins to form large protein complexes [25]. Tetraspanins modify diverse cellular functions, including integrin-mediated cell adhesion, migration, signal transduction and integrin trafficking [26-28]. A fourth reason to investigate β1 integrin interaction with lipid rafts in monocytes was our observation that

ectopic expression of the tetraspanin CD81 in U937 cells increased integrin-mediated adhesion to fibronectin in cells exposed to shear force. We hypothesized that CD81 expression might regulate integrin localization in lipid rafts required for lipid raft-mediated endocytosis.

Section 1.2 Review of the literature

1.2.1 Lipid rafts

Lipid rafts are specialized membrane microdomains that are detergent-resistant [4]. They are enriched in cholesterol, and glycosphingolipids, and accumulate glycosylphosphatidylinositol (GPI)-anchored proteins, doubly-acylated signal molecules and palmitoylated proteins [7]. The high content of cholesterol and saturated lipids renders lipid rafts highly-ordered and permits them to undergo lateral mobility in a more fluid disordered membrane bilayer comprised predominantly of unsaturated lipids [29, 30]. The strong affinity of glycolipid for cholesterol and resulting ordered phase acts as a molecular sieve to admit acylated proteins while excluding the majority of transmembrane proteins. Individual rafts are estimated to be between 25nm and 50nm in size and to contain no more than ten to thirty proteins [6]. Therefore lipid rafts are by definition highly heterogeneous in protein composition. By separating signaling molecules from each other, lipid rafts are thought to effectively dampen the unregulated initiation of signaling cascades. For signaling to proceed lipid rafts must coalesce into

larger assemblies thereby enabling signaling molecules to reach effective molarity. Coalescence is regulated through interactions with multivalent ligands [7].

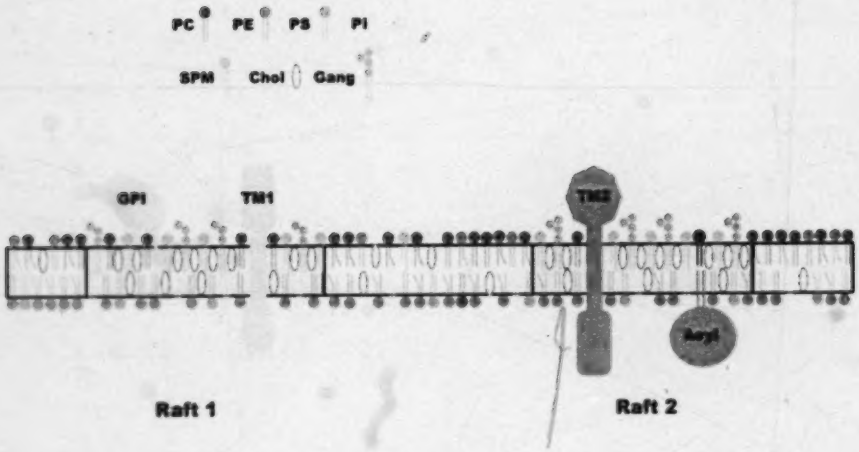

Figure I. Structure of lipid rafts. Lipid rafts (blue bilayer) are specialized membrane domains containing high concentrations of cholesterol, sphingomyelin, and gangliosides. They are also enriched in phospholipids that contain saturated fatty acyl chains (straight lines in lipid tails). This composition results in lateral phase separation and the generation of a liquid-ordered domain. Bulk plasma membrane (gray) contains less cholesterol, sphingomyelin, and gangliosides, and more phospholipids with unsaturated acyl chains. As a result, it is more fluid than lipid rafts. Not all lipid rafts have the identical protein or lipid composition (Raft 1 vs. Raft 2). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol, SPM, sphingomyelin, Chol, cholesterol; Gang, gangliosides. [31]

A major function of lipid rafts is to form platforms for compartmentalized signaling in response to receptor ligation. When receptors are activated through ligand binding it has been suggested that they oligomerize thereby increasing their affinity for lipid rafts. Receptor oligomerization would cluster rafts exposing receptors to new membrane micro-environments where their phosphorylation status could be regulated by local kinases and phosphatases, thereby initiating signaling [7]. For example, upon engagement by antigen, the zeta subunit of the T cell receptor complex segregates into lipid rafts where it associates with Src family kinases and initiates downstream signals required for T-lymphocyte activation and differentiation [32]. Conditions that disrupt T-cell lipid raft structure, abolish the earliest steps of T-cell activation indicating the importance of raft integrity for T-cell receptor signal transduction [33]. Similarly, lipid raft function is a requirement for B-cell receptor signaling, where the receptor, upon antigen binding, associates with lipid rafts containing the Src family kinase Lyn. Exclusion of the phosphatase CD45R from lipid rafts, is thought to allow Lyn to phosphorylate the receptor thereby initiating signaling [34].

1.2.2 Lipid raft-mediated endocytosis

Not only are lipid rafts found abundantly on the plasma membrane, but they are also highly enriched in recycling endosomes [35]. Additionally, lipid rafts have been implicated in sorting lipids and proteins in secretory and endocytic pathways [5]. How the clustering of membrane proteins triggers the formation of endocytic vesicles and the

consequences for endocytosis are questions of current importance.

Endocytosis is a process whereby cells internalize molecules into transport vesicles derived from the plasma membrane [1]. Multiple endocytic pathways exist, of which the best understood involves the protein clathrin. On the plasma membrane, clathrin oligomerizes to form clathrin-coated membrane invaginations that recruit cell-surface receptors for internalization. The mechanism by which clathrin-coated pits pinch off to form an endocytic vesicle requires multiple highly-regulated steps [36]. By inhibiting clathrin-mediated endocytosis through the introduction of dominant-negative clathrin mutants, it was realized that cells possess additional non-clathrin mediated endocytic pathways [37]. At least two complementary endocytic mechanisms have been identified which do not require clathrin. The first is caveolar endocytosis and the second is macropinocytosis. The extent to which lipid rafts are involved in these mechanisms remains to be elucidated [1].

Caveolae are non-coated small flask-shaped plasma membrane invaginations and, like lipid rafts, have high cholesterol and glycosphingolipid content. They aid in various signal transduction events and when stimulated, bud off from the membrane to form endocytic vesicles [38] which internalize in a dynamin-dependent pathway [39]. Caveolae are viewed as a specialized subdomain of lipid rafts and are formed by the oligomerization of caveolin-1 [40], a protein thought to stabilize their flask-like structure [29]. The presence of caveolin-1 distinguishes caveolae from lipid rafts. Caveolae were initially believed to undergo constitutive endocytic trafficking and a few researches

argued that lipid raft and caveolar endocytic pathways were indistinguishable [39]. However, recent data suggest that caveolae are highly stable membrane compartments [38] and that caveolin-1 is actually a negative regulator of caveolar trafficking [41]. In spite of this, caveolae can still internalize in response to specific stimuli by recruiting dynamin and overcoming the negative caveolin-1 regulation [29]. These studies have raised the possibility that it is the lipid raft component of caveolae that is responsible for the endocytosis of stimulated caveolae and not caveolin-1 [29].

Many cells, including human lymphocytes, blood monocytes, several cancers and transformed cell lines, fail to express, or express very low levels of caveolin-1. Cells that lack caveolin-1 do not form caveolae, but are still capable of clathrin-independent endocytosis [42]. In fact, GPI-anchored proteins, known residents of lipid rafts, endocytose into recycling endosomes using a non-clathrin, non-caveolar and dynamin-independent pinocytic pathway [11]. Similarly, clathrin-independent endocytosis occurs in B-lymphocytes which lack caveolin-1 where the B-cell receptor (BCR) sorts into a distinct lipid raft population to initiate the BCR-antigen mediated internalization [14]. The relative contribution of clathrin-dependent and independent endocytosis is reliant on both the receptor and the cell type. In B-cells, the majority of antigen-BCR complexes internalize in a clathrin-dependent manner and are delivered to intracellular compartments which are distinct from those targeted by lipid rafts [43]. These studies suggest lipid rafts contain all the necessary machinery to stimulate endocytosis and to direct proteins to distinct subcellular locations without the formation of caveolae through caveolin-1. However, how lipid raft-mediated endocytosis is regulated in caveolin-1 null

cells, remains unclear.

One of the earliest indications that lipid rafts mediate endocytosis of membrane proteins was the observation that the GPI-anchored lipid raft protein CD59, is excluded from clathrin-coated pits and trafficks to the Golgi independently of clathrin [44]. When cholesterol was lowered by exposure to the cholesterol-sequestering agent filipin, transport of CD59 to the Golgi was inhibited whereas clathrin-mediated endocytosis was unaffected. Lipid raft-associating proteins incorporate into a distinct class of clathrin-independent endosome that is devoid of markers of classical early and recycling endosomes [45]. Therefore, lipid rafts characterize unique endocytic vesicles that traffick in a pathway which is distinct from that of classical recycling endosomes.

Many bacterial toxins and viruses exploit lipid rafts to ensure their internalization into the cytoplasm. For example the filoviruses *Ebola* and *Marburg* concentrate in lipid rafts where they induce endocytosis [9]. One well-known bacterial toxin that utilizes lipid rafts for endocytosis is cholera toxin. Cholera toxin consists of an A subunit which contains the enzymatic activity and a pentameric B subunit which binds the glycosphingolipid, GM1, a major constituent of lipid rafts [10]. Clustering of GM1 by the B subunit coalesces lipid rafts promoting endocytosis of the toxic A subunit [10, 46]. In fact, interaction with the fluorescently-labeled cholera toxin-B subunit (CtxB) is sufficient to coalesce lipid rafts and other raft-associated proteins into patches and stimulate GM1 endocytosis in T-lymphocytes [19, 47]. Cholesterol, a major constituent of lipid rafts, is required for both coupling cholera toxin to GM1 and for intracellular

trafficking. Consequently, lipid rafts appear to contain all the machinery required to effect endocytosis [46].

In addition to their sorting and trafficking functions, lipid rafts may play a critical role in organizing changes in the underlying membrane actin cytoskeleton required for membrane budding, vesicle trafficking and cell polarity [48-50]. Studies of the lipid raft proteome demonstrate the presence of the cytoskeletal proteins γ and β actin, the actin cross-linking protein α-actinin, and cytoskeletal assembly proteins of the Rac family including, Rac1, Rac 2 and Rac 3 [20]. Rac proteins are members of the Rho family GTPases which are key regulators of the actin cytoskeleton [51]. Each member is believed to regulate different cytoskeletal functions, Rac regulates membrane ruffle formation, whereas RhoA controls stress fiber formation at focal adhesion points and Cdc42, another family member, is responsible for filopod extension [52]. Signaling molecules that associate with lipid rafts traffick along F-actin between exocytic and endocytic compartments [53]. The presence of actin filaments and actin-regulating proteins in lipid rafts suggests that lipid rafts can directly manipulate the organization of the actin cytoskeleton allowing for appropriate delivery of active receptors to specific lipid raft sites. The ability of lipid rafts to order spatial signaling allows cells to redistribute raft domains on the cellular membrane. For instance, it was shown that migrating and chemotaxing cells accumulate distinct lipid raft types in the leading edge and uropod, in an actin-dependent manner, allowing cells to adopt a polarized phenotype. Redistributing lipid raft-associating chemotactic receptors to the leading edge allows cells to amplify chemo-attractant intracellular signals and orient themselves to move in a

chemo-attractant gradient [49, 50]. Therefore, it appears likely that lipid rafts play a key role in cytoskeleton organization.

Cytoskeleton interaction with lipid rafts also plays an important role in signal transduction by regulating the association between proteins and lipid rafts. For example, Fc receptors are high affinity receptors for immunoglobulins on numerous cell types, including monocytes, that play fundamental roles in immune recognition and host defense [54]. Cross-linking FcεRI, an IgE Fc receptor, promotes association with lipid rafts through a mechanism which is negatively regulated by F-actin. Disrupting F-actin strengthens interactions between FcεRI and Lyn, prolonging tyrosine phosphorylation of the receptor [55]. Therefore, the cytoskeleton is responsible for ensuring both the association and disassociation of signaling proteins with lipid rafts.

Using a proteomic approach we demonstrated that lipid rafts of THP1 monocytic cells also contain proteins involved in vesicle formation, protein trafficking including the Rab family of GTPases, and the membrane fusion protein N-ethylmaleimide- sensitive fusion protein (SNARES) [20]. This suggests that vesicular transport of lipid rafts is of importance for monocyte function. Cells of the monocytic lineage must extravasate in order to infiltrate injured or infected tissues. Monocytes therefore require mechanisms to regulate adhesion to vessel wall components and to facilitate their migration into tissues. Interactions between integrins and lipid rafts could be of particular importance to monocytes because, in order to successfully extravasate, they must resist shear forces, make rapid changes in cell adhesion and conserve and recycle integrins.

1.2.3 Integrins

Integrins are a large family of heterodimeric transmembrane glycoproteins composed of one α and one β subunit. The specificity of integrin ligand binding is established by combining various α and β subunits [56]. For example, α5β1 integrin binds fibronectin whereas α4β1 integrin binds to a different site on fibronectin and also to vascular adhesion molecule 1 (VCAM-1) [57]. When integrins bind their ligands, they cluster and initiate signaling cascades which regulate fundamental processes such as gene transcription, cell survival, cell motility and cytoskeletal reorganization [58]. Additionally, integrins play a key role in T-lymphocyte immune cell maturation by acting as an anchorage for retention in the thymus and as recognition molecules for homing specificity [59]. Importantly, integrins are responsible for mediating cell-matrix and cell-cell adhesion in a wide range of biological contexts. For example, integrins α4β1 and α4β7, upon activation by chemokine receptors, serve as rolling receptors on T-lymphocytes which slow and bind T-cells to vascular walls allowing their migration across the vessel wall at sites of inflammation [57].

Integrins link the cell cytoskeleton, to the ECM. A defining characteristic of integrins is their ability to re-organize the actin cytoskeleton in response to external cues. This re-organization is required for effective cell-substrate and cell-cell adhesion and migration. The two main integrins expressed on monocytes, α4β1 and α5β1, modulate the cytoskeleton by interacting with cytoskeletal proteins such as paxillin, talin and vinculin that serve as scaffolds to recruit signaling molecules. The α4 subunit, is the only α

subunit, that directly associates with the adaptor molecule paxillin, whereas the β1 subunit of both α4β1 and α5β1 associate with the actin-binding proteins talin and vinculin [60]. Therefore, the ability of the integrins to organize cytoskeleton proteins, allows integrins to sense external clues, activate downstream signals and modulate the cytoskeleton collectively termed "outside-in" signaling. Integrins are major effectors of cell adhesion, and initiate a variety of cell survival signaling cascades in response to ECM ligation [61]. Integrins can also be regulated by intracellular signals and modulate their ligand binding ability termed "inside-out" signaling [62]. Inside-out signaling can regulate affinity, conformational changes which alter the binding capability of the heterodimer, and avidity, clustering the heterodimers into multimers, to induce integrin activation [62]. Therefore, integrins play important roles in cell response to both internal and external cues.

Lipid raft integrity is a requirement for leukocyte integrin adhesion [24] and signaling. Engagement of the integrins LFA-1 (αLβ2), α4β1 and α6β4 induces aggregation and stimulates integrins to redistribute into lipid rafts, suggesting lipid rafts play a key role in positively regulating integrin function and signaling [19, 63, 64]. Inactive integrins and non-raft associating integrins are excluded from lipid rafts by cytoskeletal restraints [19]. Reciprocally, clustering the lipid raft marker GM1 causes β1 integrins to associate with lipid rafts [47], supporting a role for integrin-raft interaction in integrin-function.

Internalization of integrins is believed to be a requirement for cell motility. For instance, calcium-dependent recycling has been observed in neutrophil migration on fibronectin,

where α5β1 integrins are endocytosed at the trailing edge and traffic to the leading edge within endocytic recycling compartments [65]. However, although integrins can undergo internalization upon engagement the mechanisms involved remain to be elucidated.

1.2.4 Tetraspanins

Integrin association with lipid rafts may be mediated through interactions with members of the tetraspanin family. Tetraspanins are a family of 32 surface proteins that span the cell membrane four times [25]. Tetraspanins, such as CD81, bind integrins and reside in lipid raft-like domains [66]. The propensity of tetraspanins to localize to lipid rafts may explain how they facilitate integrin signaling. Whether tetraspanins a) assist the residency of associated proteins within lipid rafts b) proximate the associated proteins with lipid raft-dependent signaling mechanisms or c) regulate interactions between lipid rafts and the cytoskeleton are central questions that have yet to be adequately answered.

Tetraspanins consist of two extracellular domains of unequal size, one smaller intracellular domain, and four hydrophobic transmembrane domains [67]. Importantly, the tetraspanin family contains numerous conserved amino acids which distinguish them from other four membrane-spanning proteins. Typically, the family contains four to six conserved extracellular cysteines, which form two to three disulfide bridges, and several conserved polar amino residues in the transmembrane domains [25, 68]. Tetraspanins

interact directly or through secondary association with numerous membrane proteins, implicating tetraspanins in diverse cellular functions including, metastasis, adhesion, motility, immune cell activation, proliferation and differentiation [69]. Despite, their involvement in many biological functions, the main function of tetraspanins remains to be elucidated.

The tetraspanin CD81 is expressed throughout mammalian species with a highly conserved small extracellular loop, cytoplasmic and transmembrane regions, suggesting CD81 has evolutionarily conserved functions. Sequence variation is observed in the large extracellular loop which is required for species-specific protein interaction [70]. In human tissues, CD81 is widely expressed, including most hematopoietic cell lines, but is not expressed in the U937 pro-monocytic cell line [70]. CD81 is involved in numerous cellular functions and associates with a variety of surface proteins including integrin $\alpha 4\beta 1$ [71, 72], 14-3-3, a serine/ threonine binding intracellular signaling protein, [73] and on B cells, CD19, CD21 and MHC-class II surface molecules [70]. Therefore, CD81 may function as an adaptor protein, interacting with surface molecules through its large extracellular domain and recruiting intracellular signaling proteins with its cytoplasmic tails [74]. Additionally, CD81 is responsible for the recruitment of various surface receptors to lipid rafts. For instance, the presence of CD81 in lipid rafts, can activate downstream signals such as Lck through clustering [75]. Similarly, CD81 expression is required for the B-cell receptor and co-receptors CD19/CD21 complex to associate with lipid rafts to initiate downstream signaling. Tetraspanins also act as portals for pathogen entry. CD81 has been described as a putative viral receptor for the hepatitis C virus

envelope glycoprotein E2 [76] and as a receptor for the entry and amplification in hepatocytes of the malarial parasite *Plasmodium falciparum* [77].

Interestingly, tetraspanin expression has been shown to regulate integrin-mediated cell adhesion. For example, CD81 in thymocytes, activates integrin LFA-1 in a PKC dependent pathway, which in turn increases the cells ability to adhere to other cells [78]. Additionally, CD81 was shown to promote T-cell activation and proliferation by inducing signals that rapidly activate integrin LFA-1 to a state of high avidity [79]. Importantly, CD81 regulates adhesion strengthening of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrin in monocytes under shear forces [80]. However, whether tetraspanins regulate integrin trafficking has not been investigated.

Chapter 2: Materials and Methods

Section 2.1 Reagents and antibodies

Anti-α4β1 monoclonal antibody (mAb) 44H.6 was kindly given by Dr. Linda Pilarski. Alexa-555 directly conjugated 44H.6 mAb was prepared by Allan Mak using an Alexa-555 direct conjugation kit purchased from Pierce Chemicals. Anti-α5β1 mAb P1D6 was purchased from Santa-Cruz. CtxB-biotin, CtxB Alexa-594, CtxB Alexa-488, goat anti-mouse Alexa-546 antibody (Ab), goat anti-mouse Alexa-488 Ab, streptavidin Alexa-546, Fast DiO, phalloidin-FITC, transferrin Alexa-546 and lysotracker DND-99 were purchased from Molecular Probes. Goat anti-mouse F(ab)'2-FITC fragment, donkey anti-goat Cy5 Ab and goat anti-rabbit Cy5 Ab were purchased from Jackson ImmnunoResearch Laboratories. Rabbit anti-CtxB Ab was purchased from Sigma. Anti-MHC class 1 mAb 9H.1 was generated in collaboration with Biomira. The anti-β1 mAb 138 was generated in collaboration with Biomira and purified using protein A. Anti-transferrin receptor mAb CD71 were purchased from Pharmingen (BD Biosciences).

Bovine Serine Albumin (BSA), methyl-β-cyclodextrin (MβCD), poly-L-lysine, Monodansyl-cadaverin (MDC), sucrose, tyrphostin A23, cytochalasin D, staurosporine, genistein, W-7, intact fibronectin and wortmannin were purchased from Sigma.

Nocodazole, trichostatin A, PP1 and its analog PP2 were purchased from Calbiochem. Bapta-AM was purchased from Molecular Probes.

Fibronectin conjugated to biotin was prepared by Allan Mak using an ImmunoPure Sulfo-NHS-LC-Biotin-labeling kit from Pierce chemicals. Sulfo-NHS-LC-Biotin solution was added to 2 mg of fibronectin and incubated at 4°C for 2 hours. Unreacted sulfo-NHS-LC- biotin was removed by dialysis and biotinylated fibronectin was stored at 4°C.

MAb 138 F(ab) and F(ab)'2 fragments were prepared by Allan Mak using ImmunoPure F(ab) and F(ab)'2 preparation kits purchased from Pierce Chemicals. 5 mg of intact mAb 138 were incubated with immobilized papain for F(ab) fragments or pepsin for F(ab)'2 fragments for 4 hours at 37°C in a shaking water bath. Uncleaved mAb 138 and Fc fragments were removed by binding to a protein A sepharose column and F(ab) or F(ab)'2 fragments were collected from the column eluate. F(ab) and F(ab)'2 fragments were further purified by dialysis in PBS for 24 hours with three changes of the dialysis buffer followed by two additional volumes of distilled water. F(ab) and F(ab)'2 fragments were then lyophilized and resuspended in PBS.

Section 2.2 Cell culture

U937 cells transfected with a mock GFP bicistronic vector and U937 cells transfected with tetraspanin CD81-GFP biscistronic vector were kindly provided by Dr. Shoshana Levy (Stanford University). Raji cells (ATCC CCL 86), THP-1 cells (ATCC TIB 202) and HL-60 cells (ATCC CCL 240) were obtained from the American Type Culture Collection. Mono-Mac 6 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (German Collection of Microorganisms and Cell Cultures). Cells were maintained in RPMI 1640 medium containing 10% (v/v) FBS (GIBCO) supplemented with L-glutamine (Sigma) and penicillin streptomycin solution (Sigma). Cells were maintained at a cell density between 2 and 4 x 10^5 cells/mL.

Section 2.3 Fluorescence microscopy: cell-staining procedures

All cells were initially washed with 0.1% (w/v) BSA/RPMI before Ab incubation. After each Ab labeling the cells were washed 2 times with 5 mls 0.1% (w/v) BSA/RPMI. All Ab were diluted in 100 µL 0.1% (w/v) BSA/RPMI.

α4β1 integrin location in U937 cells: cells were first placed on poly-L-lysine for 15 min at 4°C, then fixed with 2% (w/v) paraformaldehyde/cytoskeletal-stablizing buffer (PFA/CSB) for 10 min at 4°C, and then permeabilized with 0.2% (w/v) TritonX100/cytoskeletal-stabilizing buffer (Triton/CSB) for 5 min at 4°C. The cells were

then incubated in 100μL aliquots of $5x10^5$ cells in 0.1% (w/v) BSA/RPMI containing 1/50 dilution anti-α4β1 mAb 44H.6 at 4˚C. Integrins were then labeled with 10 μg/ml goat anti-mouse Ab-Alexa 546 at 4˚C. Alternatively, U937 cells were labeled with 10 μg/ml Alexa-555 directly conjugated anti-α4β1 mAb 44H.6 at 4˚C and then placed on poly-L-lysine coated cover slips and fixed.

Integrin α4β1 and α5β1 cross-linking with secondary Ab: cells were incubated in 100 μL aliquots of $5x10^5$ cells in 0.1% (w/v) BSA/RPMI containing 1/50 dilution anti-α4β1 mAb 44H.6 or 5 μg/ml anti-α5β1 mAb P1D6 at 4˚C for 60 min, then cross-linked with 10 μg/ml goat anti-mouse Ab-Alexa 546 at 37˚C or 4˚C for 60 min. Cells were then stained with 10 μg/ml fast DiO to label the plasma membrane green. Cells were placed on poly-L-lysine for 15 min at 4˚C and fixed with 2% (w/v) PFA/CSB.

α4β1 and α5β1 integrin co-internalization with GM1: cells were incubated with 1/50 dilution anti-α4β1 mAb 44H.6 or 5 μg/ml anti-α5β1 mAb P1D6 at 4˚C for 60 min, then cross-linked with 10 μg/ml goat anti-mouse Ab-Alexa 488 at 4˚C for 60 min. Lastly, cells were labeled with 10 μg/ml CtxB-Alexa 594 at 4˚C for 60 min and incubated at 37˚C for 60 min. Cells were placed on poly-L-lysine and then fixed as above.

α4β1, α5β1, MHC class1 and transferrin receptor co-localization with cross-linked GM1: GM1 was first cross-linked by incubating cells with CtxB-biotin at 4°C for 60 min, then cross-linked with streptavidin-546 at 37°C for 60 min. The cells were then placed on poly-L-lysine, fixed, and permeabilized with 0.2% (w/v) Triton/CSB for 5 min at 4˚C.

Cells were then incubated with 1/50 dilution anti-α4β1 mAb 44H.6, 5 μg/ml anti-α5β1 mAb P1D6, 10 μg/ml anti-MHC class 1 mAb 9H.1 or 5 μg/ml anti-transferrin receptor mAb CD71 at 4˚C for 60 min, then fluorescently labeled with 10 μg/ml goat anti-mouse Ab-Alexa 488 at 4˚C for 60 min. MβCD treatment: the cells were treated with 15 mM MβCD for 15 min at 37˚C prior to GM1 cross-linking.

GM1 cross-linking at 37˚C or 4˚C: cells were incubated with 10 μg/ml CtxB-biotin or CtxB-Alexa 594 at 4˚C for 60 min, and then cross-linked with 10 μg/ml streptavidin-Alexa 546, or 1/150 dilution rabbit-anti CtxB at 37˚C or at 4˚C for 60 min. *CtxB ligated GM1 internalization:* cells were incubated with 10 μg/ml CtxB subunit-Alexa 594 at 4˚C for 30 min, washed then incubated at 37˚C for 60 min. Cells were then stained with 10 μg/ml fast DiO. Cells were placed on poly-L-lysine and fixed. *GM1 time lapse internalization in THP-1*: Cells were then incubated at various time points (0, 10, 20 and 60 min) at 37˚C.

mAb 138 F(ab) and 138 intact cross-linked with goat anti-mouse F(ab)'2 internalization: cells were incubated with 20 μg/ml 138 F(ab) or 138 intact mAb for 60 min at 4˚C. Following this incubation the Abs were cross-linked with 20 μg/ml goat anti-mouse F(ab)'2-FITC conjugate for 60 min at 37˚C. Cells were placed on poly-L-lysine and fixed.

Clathrin-independent endocytosis tests: cells were incubated with 10 μg/ml CtxB-biotin, 1/50 dilution anti-α4β1 mAb 44H.6, or 5 μg/ml anti-α5β1 mAb P1D6 at 4˚C for 60 min,

then cross-linked with 1/150 dilution rabbit anti-CtxB or 10 μg/ml goat anti-mouse Ab-Alexa 546 at 37˚C for 60 min. During the 37˚C incubation cells were treated with 0.45 M sucrose hypertonic solution, a known inhibitor of clathrin endocytosis [81]. As a control, cells suspended in the high sucrose hypertonic solution were also incubated with 50 μg/ml transferrin-Alexa 546 at 37˚C for 60 min, known to undergo clathrin-dependent endocytosis. Cells were then stained with 10 μg/ml fast DiO. Cells were placed on poly-L-lysine and fixed.

Fibronectin internalization at 37˚C: U937 and THP1 cells were incubated with 5 μg/ml biotinylated fibronectin at 4˚C, then cross-linked with 2 μg/ml streptavidin- Alexa 546 for 60 min at 37˚C. Cells were then stained with 10 μg/ml fast DiO. Cells were placed on poly-L-lysine and fixed.

GM1 and integrin internalization co-localization with transferrin or lysotracker: cells were incubated with 10 μg/ml CtxB-Alexa 488, 1/50 dilution anti-α4β1 mAb 44H.6 or 5 μg/ml anti-α5β1 mAb P1D6 at 4˚C for 60 min, then cross-linked with 1/150 dilution rabbit anti-CtxB or 10 μg/ml goat anti-mouse Ab-Alexa 488 at 4˚C for 30 min. The cells were then incubated with 1 μM lysotracker DND-546 or 20 μg/ml transferrin-546 at 37˚C for 60 min. Cells were placed on poly-L-lysine and fixed.

Section 2.4 Fluorescence microscopy: CD81 transfected U937 cell-staining procedures

α4β1 and α5β1 integrin cross-linking at 37˚C: cells were incubated with 1/50 dilution anti-α4β1 mAb 44H.6 or 5 μg/ml anti-α5β1 mAb P1D6 at 4˚C for 60 min, then cross-linked with 10 μg/ml goat anti-mouse Ab-Alexa 546 at 37˚C for 60 min. Cells were placed on poly-L-lysine, fixed, permeabilized and stained with 5 μg/ml phalloidin-FITC for 30 min at 4°C to label F-actin. Nocodazole washout treatment: cells were first treated with 15 μg/ml nocodazole for 60 min at 37°C and then washed twice with 0.1% (w/v) BSA/RPMI.

Nocodazole washout and cytochalasin D treatment: cells were first treated with 15 μg/ml nocodazole for 60 min at 37°C and then washed. Cells were then treated with 10 μg/ml cytochalasin D for 15 min at 37°C and washed.

Nocodazole washout with Trichostatin A treatment: cells were first treated with 100 nM trichostatin A for 24hrs at 37˚C and then washed. Cells were then treated with 12.5 μg/ml nocodazole at 37˚C for 60 min followed by washing.

Integrin capping quantitation: cells were incubated with 1/50 dilution anti-α4β1 mAb 44H.6 or 5 μg/ml anti-α5β1 mAb P1D6 at 4˚C for 60 min, then cross-linked with 10 μg/ml goat anti-mouse Ab-Alexa 546 at 37˚C for 60 min. Cells were placed on poly-L-lysine then fixed. Single optical sections of over 100 cells per sample were taken at mid-height using confocal microscopy. Cells with all integrins located within an area that was less than 50% of the total cell volume were considered to have large integrin caps. This

cell number was compared against the total number of cells to determine the percentage of cells with large integrin caps.

Section 2.5 Integrin and GM1 endocytosis quantitation

Integrin α4β1 and GM1 cross-linking: cells were incubated with 1/50 dilution anti-α4β1 mAb 44H.6, or 10 μg/ml CtxB-Alexa 594 at 4°C for 60 min, then cross-linked with 10 μg/ml goat anti-mouse Ab-Alexa 546 (α4β1) or 1/150 dilution rabbit anti-CtxB Ab (GM1) at 37°C for 60 min. Cells were then incubated with tertiary Ab: 10 μg/ml donkey anti-goat Cy5 Ab or goat anti-rabbit Cy5 Ab for 45 min at 4°C. Cells were placed on poly-L-lysine and fixed as described above. All inhibitors were diluted in 0.1% (w/v) BSA/RPMI except for Bapta-AM which was diluted in 0.1% (w/v) BSA/PBS. MβCD treatment: The U937 cells were treated with 15 mM MβCD for 15 min at 37°C prior to integrin/GM1 cross-linking at 37°C. All other inhibitors were treated during integrin/GM1 cross-linking at 37°C.

Table 1: Concentrations and conditions of inhibitors used to inhibit internalization.

Treatment	Concentration	Conditions
Cytochalasin D	10 μg/ml	60 min at 37°C
Nocodazole	15 μg/ml	60 min at 37°C
Staurosporine	1 μM	60 min at 37°C

PP1 and PP2	4 μg/ml	60 min at 37°C
Wortmannin	10 μM	60 min at 37°C
Bapta AM	50 μg/ml in 1xPBS	60 min at 37°C
Genistein	100 μg/ml	60 min at 37°C
W-7	10 μM	60 min at 37°C

Nocodazole washout treatment: cells were first treated with 15 μg/ml nocodazole for 60 min at 37°C and then washed twice prior to integrin/GM1 cross-linking at 37°C.

Section 2.6 Confocal microscopy

All experiments were performed a minimum of three times to confirm reproducibility except for integrin and GM1 internalization in MM-6 cells and fibronectin internalization in U937 cells which were only reproduced twice. Isotype Ab controls utilizing mouse mAb MOP-C, instead of the primary Ab, were completed when applicable for a negative control and to assess background noise. All experiments included a secondary Ab only (no primary mAb) negative control to set the confocal microscope for background noise. Fluorescent cells were observed and analyzed using Zeiss LSM 510 confocal laser scanning microscope with 40X objective. Excitation was produced using an argon laser for 488nm wavelength, helium neon laser for 543nm wavelength, or helium neon laser for 633nm wavelength. Cell surface distributions of fluorescent proteins were evaluated by taking Z-stack optical sections horizontally throughout the entire representative cell or

mid section through the cells. For each slide undergoing microscopic analysis, a total of six different groups of cells (usually three to five cells per group) were imaged. For each slide, three full Z-stacks were completed to reconstruct cells 3-dimensionally and three images were taken at the mid section of different cells in different locations on each slide. Images of optical sections were taken at 512 x 512 pixel resolution and were recorded digitally. Using the palette tool built into the Zeiss LSM confocal software we controlled for fluorescence pixel saturation for each channel (red and green) for each slide. The resulting images were processed using Adobe Photoshop software.

Internalization quantitation was completed by taking full Z-stack optical sections of 20 individual cells for each sample set. Using Imaris co-localization software, the fluorescent intensity of each channel (secondary and tertiary Ab) were analyzed within the volume of the entire 3-dimensional cell. Pixels that contained flourescence from both channels were compared with pixels that contained only one channel (the secondary Ab channel). Using correlation algorithms, co-localization channel correlations for the data set volume was determined for each 3-dimensional cell. All 20 data sets for each experiment were then averaged and the standard deviation was determined (error bars). The results of each sample set were then graphed using Microsoft Excel software.

Section 2.7 Flow cytometry analysis

Flow cytometry analysis of α4β1 and α5β1 integrin expression: all cells were initially washed with 0.1% (w/v) BSA/RPMI before incubation with antibody. After each antibody incubation, the cells were washed twice with 0.1% (w/v) BSA/RPMI. $1x10^6$ cells were labeled with 5 μg/ml anti-β1 mAb 138, 1/50 dilution anti-α4β1 mAb 44H.6 or 5 μg/ml anti-α5β1 mAb P1D6 for 60 min at 4°C. Cells were then incubated with 10 μg/ml goat anti-mouse Ab-phycoerythrin (PE) conjugate at 4°C for 60 min to fluorescently label the integrins. The cells were then subjected to flow cytometry analysis.

Section 2.8 Reverse transcriptase (RT)-PCR analysis of caveolin-1

RT-PCR for caveolin-1 was performed by Lorri Martin. Total RNA was extracted from 1×10^6 cells using TRIzol Reagent (Invitrogen Life Technologies). Oligo-dT-primed cDNA synthesis, using 1 μg of total RNA was synthesized in a volume of 20 μL with the SuperScript RNase H-RT cDNA synthesis kit (Invitrogen). Negative RT controls were performed on all RNA samples to ensure that the RNA was not contaminated with genomic DNA for the subsequent PCR reaction. The PCR reaction was performed with 2 μL cDNA, equivalent to 0.1 μg of RNA, in a volume of 25 μL using Taq DNA Polymerase Recombinant kit (Invitrogen) and 10 μmol/L gene-specific primers. Primers used were caveolin-1-L, 3'-CCA CCT TCA CTG CGA AAT A-5', and caveolin-1-R, 5'-

AGA TGG AAT AGA CAC GGC TCA T-3'. The PCR was run in a GeneAmp 2400 (Perkin-Elmer) with a program that had an initial 5 min denaturation at 94°C, followed by 30 cycles consisting of 30s at 94°C, 30s at primer-specific annealing temperature, 1 min at 72°C, and a final extension of 7 min at 72°C. The expected size of the amplification product was 183 bp. The U266 multiple myeloma cell line was used as a positive caveolin-1 expressing cell line [82]. Reaction products were analyzed by electrophoresis in a 2% (w/v) agarose gel containing ethidium bromide and were visualized by exposure to UV light.

Chapter 3: Results. Lipid rafts mediate β1 integrin endocytosis in monocytes.

Section 3.1 Introduction and experimental objectives

Integrins are the major cell surface proteins responsible for cell-cell adhesion, cell-matrix adhesion and cell migration. Cells require mechanisms to optimize binding to the immobilized ligands, which provide the mechanical purchase necessary for locomotory activity. Solubilization of the ECM by extracellular proteases is a consistent feature of tissue remodeling and cellular extravasation [83]. Solubilized ligands reduce the efficiency of adhesion and migration by competing with immobilized ligands for integrin binding. Therefore, cells which must optimize their adhesion to leave the blood are likely to employ mechanisms to rapidly clear integrins occupied by soluble ligands from their surface. Several studies have demonstrated that ligating integrins with monoclonal antibodies and further cross-linking with secondary antibodies at 37°C induces endocytosis [16-18]. However, the processes involved have not been defined. Monocytes require mechanisms to optimize their adhesion to components of the vessel wall in order to resist shear force and subsequently to migrate into regions of inflammation. We reasoned therefore, that monocytes would constitute a useful model system for the investigation of integrin internalization. We had observed that, although adhesion of monocytes to fibronectin under static conditions resisted treatment with

MβCD, adhesion under shear force was particularly sensitive to inhibition, indicating that lipid rafts play a critical role in strengthening integrin-mediated adhesion under temporal constraint. We hypothesized that if integrins associate with lipid rafts in order to effect rapid changes in cell adhesion then clustering integrins with soluble antibodies that mimic soluble ligands might trigger lipid raft-dependent internalization. To confirm integrin endocytosis was lipid raft-mediated we needed to rule out both caveolar- and clathrin-mediated endocytosis as possible mechanisms of integrin internalization. Also, since monocytes express the Fc receptors: FcγRI and FcγRII which bind to and internalize [84] clustered IgG2a or IgG2b/1 [85] we needed to exclude Fc receptor-mediated endocytosis as the mechanism of integrin internalization.

To determine whether lipid raft-mediated integrin endocytosis occurred in other monocytic cell lines, we investigated the AML promonocytic cell line THP-1 and the mature monocytic cell line Mono Mac-6. To investigate whether it occurred in non-monocytic cells we examined the promyelocytic cell line HL-60 and the B-lymphocyte cell line Raji. To decipher whether internalized integrins and lipid rafts are directed to distinct subcellular locations or endocytic pathways after internalization we looked for co-localization with endosomes or lysosomes. Finally, we asked wether similar mechanisms were involved in integrin and GM1 endocytosis such as the cytoskeletal requirements and signaling effector proteins to look for evidence of a similar pathway.

Section 3.2 Cross-linking α4β1 and α5β1 integrins with secondary antibody triggers integrin endocytosis.

When the integrin α4β1 was ligated with a monoclonal antibody at 4˚C and visualized with the confocal microscope, it was found to be distributed evenly over most of the plasma membrane (Figure 1). However, when cells were incubated with the anti-α4β1 mAb 44H.6 or with the anti-α5β1 mAb P1D6 at 4˚C, then cross-linked with goat anti-mouse Ab-Alexa 546 and incubated at 37˚C for 60 min, the integrins clustered and began to internalize (Figure 2C and 2D). When the integrins α4β1 and α5β1 were ligated with mAb at 4˚C and cross-linked with the secondary antibody at 4°C the integrins were still evenly distributed on the plasma membrane and did not internalize (Figure 2A and 2B). This suggests integrin internalization is an active process requiring metabolic activity and membrane fluidity. Figure 2E represents close-ups of boxed cells from Figures 2A to 2D to help demonstrate the differences observed between integrins cross-linked at 4°C and at 37°C. To differentiate the internalized molecules from those remaining at the plasma membrane we used the nonspecific lipid dye, DiO to label the plasma membrane. This worked well in most experiments. However, occasionally the DiO also labeled internal membranes. We defined internalized integrins as integrin clusters that were no longer in contact with the cell's periphery. Interestingly, there was substantial variation in the amount of integrin expression and internalization observed between individual cells. However, by observing greater than 25 cells and reconstructing 3-dimensional images of individual cells we found that the majority of the cells contained at least some internal integrin clusters.

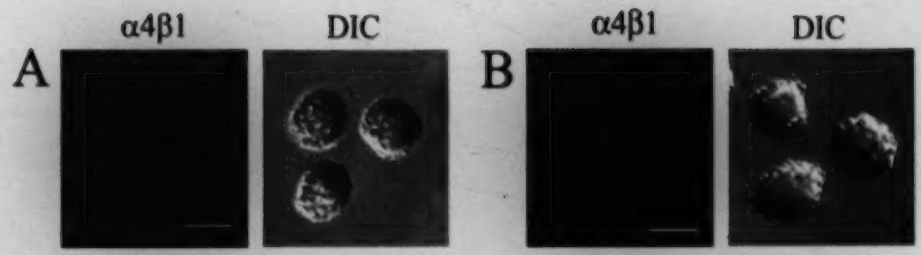

Figure 1. Location of α4β1 integrin on U937 cells. Cells were place on poly-L-lysine, fixed, permeabilized, and incubated with anti-α4β1 mAb 44H.6 (A) at 4°C. Integrins were then labeled with goat anti-mouse Ab-Alexa 546 at 4°C. Alternatively, the cells were labeled with Alexa-555 directly conjugated anti-α4β1 mAb 44H.6 at 4°C (B) and then placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panels (A & B) show that α4β1 integrins localize to the cell membrane and diffusely cover most of the cell membrane. α4β1 integrin, red. Bars, 10 μm.

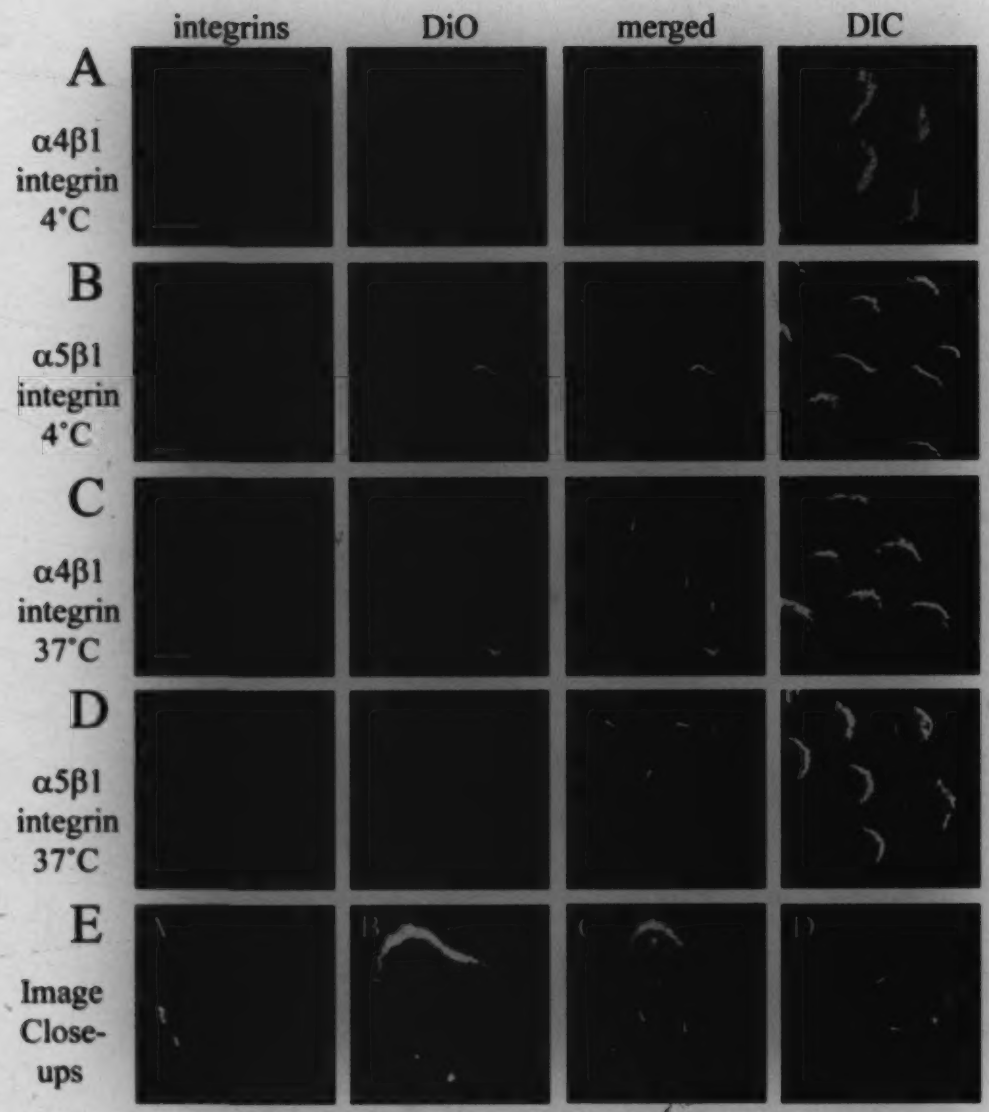

Figure 2. Integrins α4β1 and α5β1, when cross-linked with a secondary antibody at 37°C, patch and begin to internalize in U937 cells. Cells were incubated with anti-α4β1 mAb 44H.6 (A & C) or anti-α5β1 mAb P1D6 (B & D) at 4°C, then cross-linked with goat anti-mouse Ab-Alexa 546 at 4°C (A & B) or at 37°C (C & D) for 60 min. Cells were then stained with fast DiO to label the plasma membrane green. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Top panels (A & B) show 4°C cross-linked α4β1 and α5β1 integrins localize to the plasma membrane. Bottom panels (C & D) show 37°C cross-linked α4β1 and α5β1 integrins cluster and internalize. Panel E shows image close-ups of boxed cells from each panel (A-D). α4β1 and α5β1 integrins, red; DiO, green. Bars, 10 μm. Arrows indicate points of integrin internalization.

Section 3.3 Cross-linked integrins endocytose via lipid rafts

Activation of the integrin αLβ2 resulted in its redistribution into the lipid rafts of T-cells [19]. Therefore, we wanted to determine whether the two major integrins of the U937 monocytic cell line, α4β1 and α5β1, co-localized with lipid rafts upon clustering. When we cross-linked the integrins at 37°C and labeled the ganglioside GM1, with CtxB conjugated to Alexa 594, the integrins clustered into patches on the cell membrane with some clusters co-localizing with GM1 (Figure 3A and 3B). Additionally, many internal integrin clusters also co-localized with GM1 suggesting that integrins endocytose through a lipid raft-mediated mechanism. Close-ups of selected cells (boxed) demonstrate sites of integrin/GM1 co-localization (Figure 3C). This raised the question whether integrin clustering triggers a raft-based endocytic mechanism through the coalescence of lipid rafts. Integrins that failed to co-localize with GM1 may represent non-lipid raft associating integrins, since non-functional integrins are thought to be excluded from lipid rafts [19].

Having shown that many internalized integrins co-localize with GM1, we wondered whether cross-linking GM1 with multivalent proteins would co-cluster β1 integrins, and if so whether β1 integrin co-clustering was required for GM1-induced endocytosis. To investigate this, we cross-linked the ganglioside GM1 and asked whether α4β1 or α5β1 co-clustered, whether clustering induces internalization of GM1 and whether the integrins co-internalized. Cells were incubated with CtxB-biotin at 4°C, then cross-linked with streptavidin-Alexa 546 at 37°C. CtxB is a pentameric protein and therefore

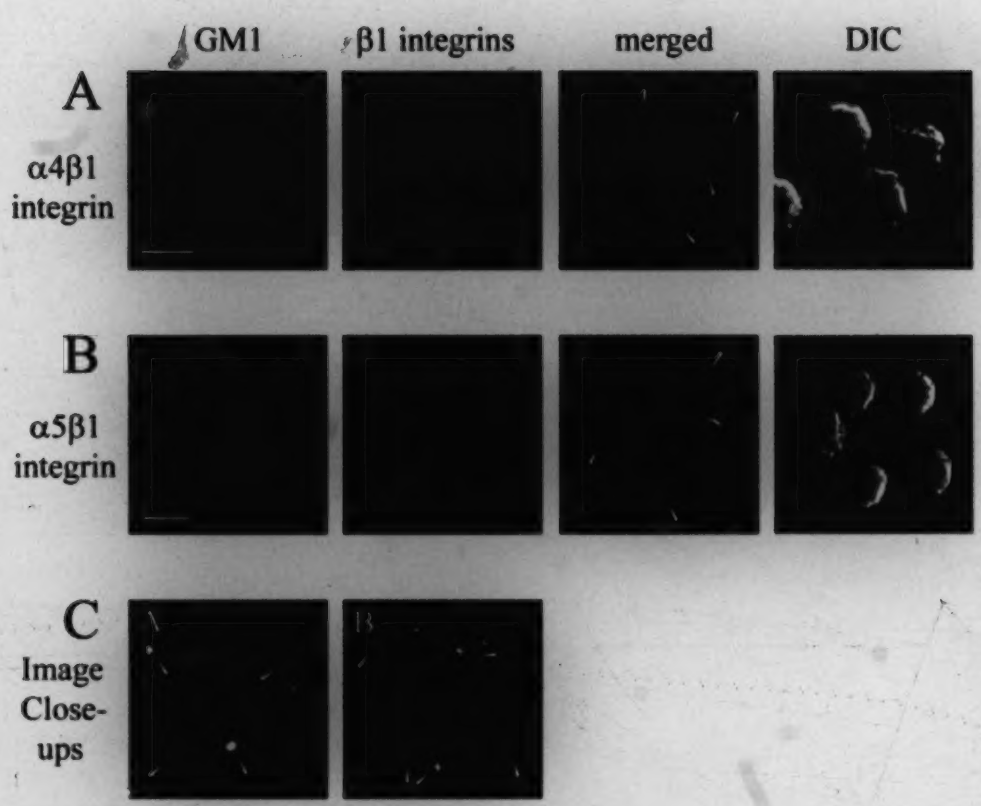

Figure 3. Cross-linked α4β1 and α5β1 integrins associate with and internalize with GM1. Cells were incubated with anti-α4β1 mAb 44H.6 (A) or anti-α5β1 mAb P1D6 (B) at 4˚C, then cross-linked with goat anti-mouse Ab-Alexa 488 at 4˚C. Subsequently, the cells were labeled with Ctx-594 at 4˚C. Cells were incubated at 37˚C, andthen placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panels (A & B) show that integrins α4β1 and α5β1 upon cross-linking at 37˚C co-internalize GM1. Panel C shows image close-ups of boxed cells from each panel (A & B). α4β1 and α5β1 integrins, green; GM1, red. Bars, 10 μm. Arrows indicate points of integrin/GM1 co-localization.

stimulates GM1 clustering but to cluster lipid rafts and their associating proteins we further clustered GM1 with streptavidin. Since, streptavidin is a tetrameric protein each streptavidin molecule is able to bind to four biotin molecules. The cells were then fixed, permeabilized and labeled with primary anti-α4β1 mAb 44H.6 or anti-α5β1 mAb P1D6 mAb followed by fluorescent secondary antibodies at 4°C. Using confocal microscopy to observe 3-dimensionally reconstructed cells, we observed that in the majority of the cells, unengaged α4β1 and α5β1 integrins co-capped on the cell surface with cross-linked GM1, suggesting that they co-cluster with lipid rafts (Figure 4A and 4B). However, although GM1 internalized, α4β1 and α5β1 integrins remained on the cell surface indicating that lipid rafts are self-sufficient in endocytic machinery. Co-capping of unoccupied integrins with GM1 indicate that integrins have a constitutive affinity for clustered lipid rafts. However, that affinity is not sufficient to incorporate unoccupied integrins into endosomes suggesting that sorting mechanisms separate endocytosing lipid rafts from unoccupied integrins. Such a mechanism would probably be necessary to prevent wholesale depletion of integrins.

MHC class I is a transmembrane heterodimeric protein that presents peptides to T cell receptors and is not thought to have a high affinity for lipid rafts. The transferrin receptor imports transferrin into cells through incorporation into endosomes within clathrin-coated pits and is specifically excluded from lipid rafts. MHC class I and the transferrin receptor therefore serve as specificity controls for lipid-raft mediated uptake of integrins [19, 86, 87]. We therefore, asked whether cross-linking GM1 would co-cluster MHC class I or the transferrin receptor. Following GM1 clustering with biotinylated CtxB and

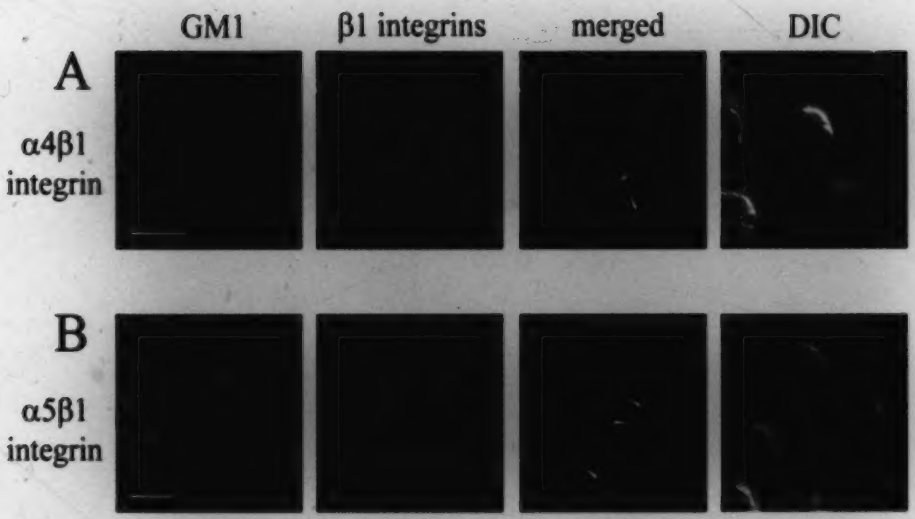

Figure 4. α4β1 and α5β1 integrins do not internalize with cross-linked GM1 indicating that monocytic lipid rafts contain the machinery for endocytosis which is triggered by clustering the rafts. Cells were incubated with CtxB-biotin at 4°C, then cross-linked with streptavidin Alexa-546 at 37°C. The cells were placed on poly-L-lysine, fixed and permeabilized. The cells were then incubated with anti-α4β1 mAb 44H.6 (A) or anti-α5β1 mAb P1D6 (B) at 4°C, and then labeled with goat anti-mouse Ab-Alexa 488 at 4°C. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panels (A & B) show that α4β1 and α5β1 integrins do not co-internalize with cross-linked GM1. α4β1 and α5β1 integrins, green; GM1, red. Bars, 10 μm. Arrows indicate points of GM1 internalization.

streptavidin, cells were fixed, permeabilized, and incubated with either anti-MHC class I mAb 9H.1 or anti-transferrin receptor mAb CD71 at 4°C, and further labeled with goat anti-mouse Ab-Alexa 488 at 4°C. MHC class I and the transferrin receptor did not co-cluster with GM1 (Figure 5A and 5B) indicating that integrins have a constitutive affinity for GM1 which exceeds that of the other two cell surface receptors.

Section 3.4 Integrin association with lipid rafts is cholesterol-dependent

Cholesterol is a major constituent of lipid rafts and removal of cholesterol is known to disrupt lipid rafts. Therefore, we wanted to determine what happens to both GM1 and β1 integrin distribution when cells are depleted of cholesterol. To accomplish this we treated the cells with MβCD. MβCD treatment removes up to 60% of all cellular cholesterol from the plasma membrane. Cells were treated with 15 mM MβCD for 15 min at 37°C and washed. After MβCD treatment, GM1 was cross-linked with CtxB-biotin and streptavidin at 37°C, the cells were then fixed, permeabilized, and both integrin α4β1 and α5β1 were labeled fluorescently at 4°C as described in the materials and methods (Section 2.3). Using confocal microscopy we observed that MβCD treatment resulted in a diffuse distribution of both α4β1 and α5β1 integrins on the plasma membrane and inhibited GM1 internalization (Figure 6A and 6B). MβCD treatment disrupted integrin co-localization with GM1, suggesting that the association requires interaction with GM1 in the context of lipid rafts.

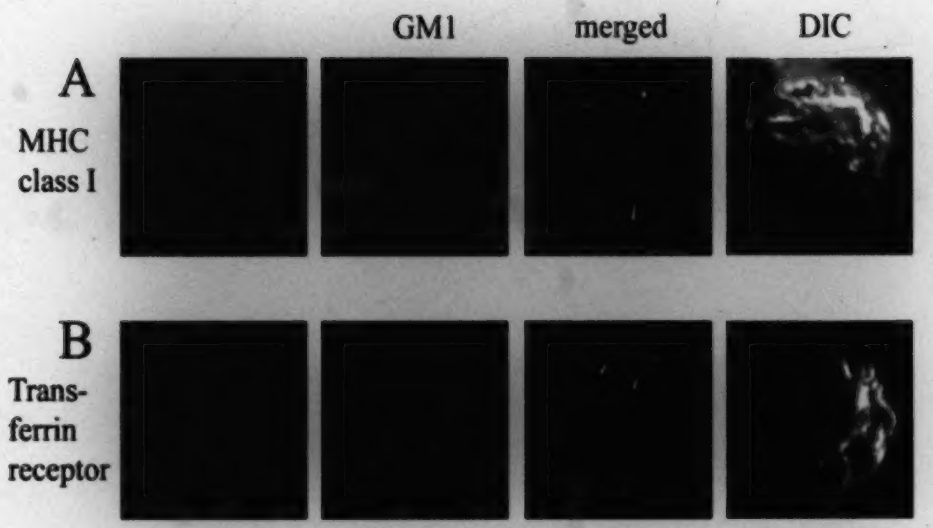

Figure 5. MHC class I and the transferrin receptor do not co-cluster with cross-linked GM1. Cells were incubated with CtxB-biotin at 4°C, then cross-linked with streptavidin Alexa-546 at 37°C. Cells were placed on poly-L-lysine, fixed and permeabilized. Cells were then incubated with anti-MHC class I mAb 9H.1 (A) or anti-transferrin receptor mAb CD71 (B) at 4°C, and then cross-linked with goat anti-mouse Ab-Alexa 488 at 4°C. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panels show MHC class 1 (A) and the transferrin receptor (B) do not co-cluster cross-linked GM1. Therefore, MHC class 1 and the transferrin receptor have a low affinity for lipid rafts. MHC class 1 and the transferrin receptor, green; GM1, red. Bars, 10 μm. Arrows indicate points of GM1 internalization.

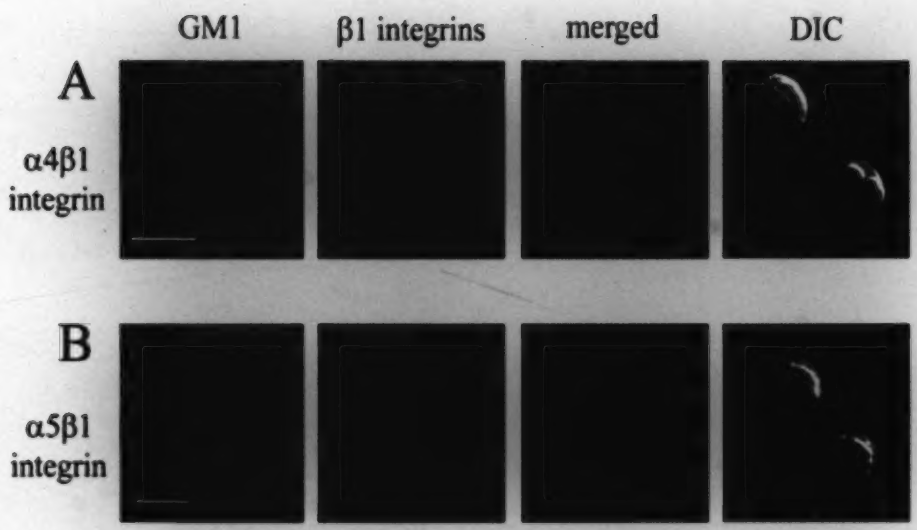

Figure 6. Cross-linked GM1 does not internalize and does not co-localize with α4β1 and α5β1 integrins in MβCD treated cells. Cells were first treated with 15 mM MβCD for 15 min at 37°C, then incubated with CtxB-biotin at 4°C, and then cross-linked with streptavidin Alexa-546 at 37°C. Cells were placed on poly-L-lysine, fixed and permeabilized. The cells were then incubated with anti-α4β1 mAb 44H.6 (A) or anti- α5β1 mAb P1D6 (B) at 4°C, and then cross-linked with goat anti-mouse Ab-Alexa 488 at 4°C. Single optical sections were taken of cells shown at mid-height using confocal microscopy. Panels (A & B) show that cross-linked GM1 does not internalize and does not co-localize with α4β1 or α5β1 integrin in MβCD treated cells. This concludes that cholesterol is a requirement for GM1 endocytosis and cholesterol depletion disrupts integrin association with lipid rafts. α4β1 and α5β1 integrins, green; GM1, red. Bars, 10 μm.

Section 3.5 Is GM1 and integrin endocytosis Fc receptor independent?

Monocytes express FcγRI and FcγRII which are Fc receptors for immunoglobulin [85]. To exclude Fc receptor activation as a possible mechanism for integrin and GM1 endocytosis, GM1 was cross-linked with streptavidin or with rabbit anti-CtxB Ab which contains an Fc region for comparison purposes, as described in the materials and methods (Section 2.3). To label the plasma membrane we stained the cells with fast DiO. Using confocal microscopy, we observed that cross-linking GM1 at 4˚C fails to internalize GM1 indicating cells must be at physiological temperatures to actively internalize GM1 (Figure 7A). However, on cross-linking GM1, with either a secondary antibody (rabbit anti-CtxB) or with streptavidin at 37˚C, GM1 clusters and internalizes equally well in both samples (Figure 7B and 7C). This indicates that lipid raft endocytosis does not require Fc receptor activation. Figure 7D represents close-ups of boxed cells from Figures 7A to 7C to help demonstrate the differences observed between GM1 cross-linked at 4°C and at 37°C.

CtxB is a pentameric molecule and can therefore cluster GM1 in the absence of a secondary cross-linking agent. To determine whether clustering with CtxB alone is sufficient to promote endocytosis, cells were incubated with CtxB conjugated to Alexa 594 at 4˚C, washed, then incubated at 37˚C. Cells were finally stained with fast DiO. Figure 8 shows ligation of GM1 with CtxB is sufficient to induce lipid-raft mediated endocytosis.

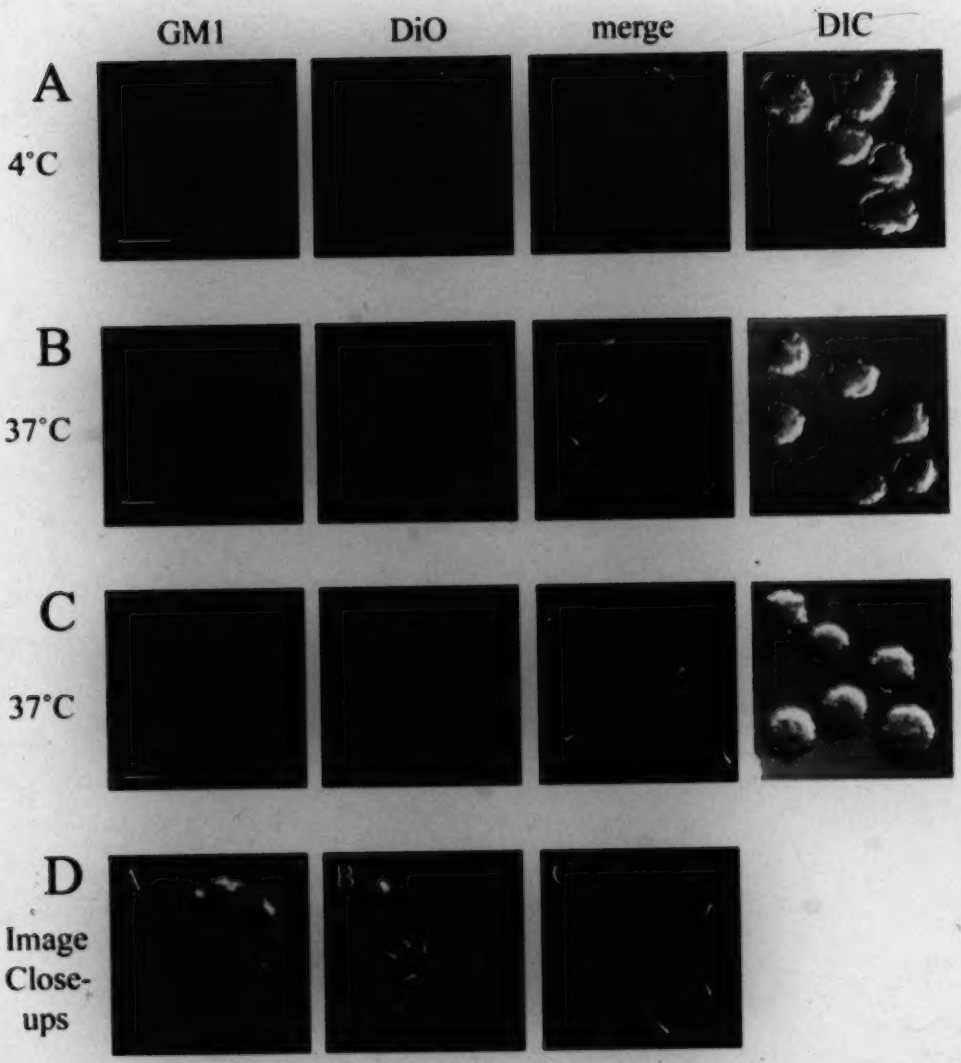

Figure 7. GM1 internalization is Fc receptor activation independent. Cells were incubated with CtxB Alexa-594 (A & B) or CtxB-biotin conjugated (C) at 4°C, then cross-linked with rabbit anti-CtxB Ab (A & B) or streptavidin Alexa-546 (C) at 4°C (A) or 37°C (B & C). Cells were then stained with fast DiO to label the plasma membrane green. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Top panel (A) shows GM1 cross-linked at 4°C remains located on the plasma membrane. Panel B shows cross-linked GM1 with rabbit anti-CtxB clusters and internalizes. Panel C shows GM1 cross-linked with streptavidin also undergoes internalization, indicating GM1 endocytosis is not a result of Fc receptor activation. Panel D shows image close-ups of boxed cells from each panel (A-C). GM1, red; DiO, green. Bars, 10 μm. Arrows indicate points of GM1 internalization.

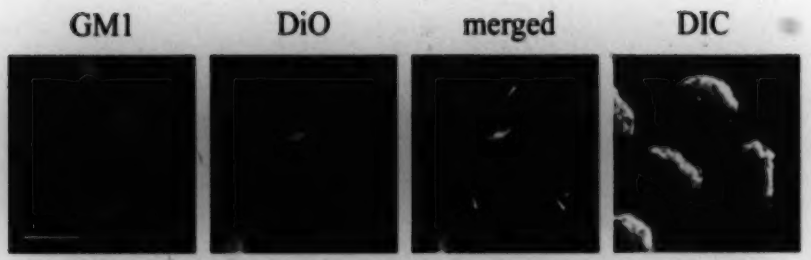

Figure 8. GM1 ligated with CtxB patches and internalizes in U937 cells. Cells were incubated with CtxB-Alexa 594 at 4°C, washed then incubated at 37°C. Cells were then stained with fast DiO to label the plasma membrane green. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Figure shows labeling GM1 with CtxB alone is sufficient to induce lipid-raft mediated endocytosis, indicating that pentameric CtxB does have some lipid raft clustering capability. GM1, red; DiO, green. Bars, 10 μm. Arrows indicate points of GM1 internalization.

To confirm that integrin-mediated endocytosis is also Fc receptor independent in U937 cells, β1 integrins were ligated with F(ab) fragments of the anti-β1 integrin mAb 138 at 4°C and cross-linked with a goat anti-mouse $F(ab)'_2$ secondary antibody at 37°C. For comparison, U937 cells were ligated with intact mAb 138 at 4°C and cross-linked with a goat anti-mouse $F(ab)'_2$ secondary antibody at 37°C. In both cases, we observed β1 integrin cluster into patches and internalize (Figure 9A and 9B). Therefore lipid raft mediated endocytosis does not require Fc receptor engagement, or signaling, and can be triggered simply by clustering.

Section 3.6 GM1 and integrin endocytosis is cell-line specific

To investigate whether clustering GM1 or β1 integrins would promote endocytosis in cells of other lineages or is a specific property of the U937 monocytic cell line, we cross-linked GM1 and the α4β1 integrin in THP1, MonoMac-6, HL-60 and Raji. We cross-linked GM1 and α4β1 with primary and secondary antibodies and incubated at 37°C as described in the materials and methods (Section 2.3). Using confocal microscopy we observed cells some of which were reconstructed 3-dimensionally. As indicated in Figure 10, clustering GM1 induced endocytosis in THP-1 (Figure 10A), but not in the other cell lines (Figures 10B, 10C and 10D). The inability to induce endocytosis in the more differentiated and macrophage-like MonoMac-6 cell line compared to the less differentiated U937 and THP1 cell lines was unexpected and may reflect a functional switch in the utilization of lipid rafts for chemokine-dependent signaling in the more

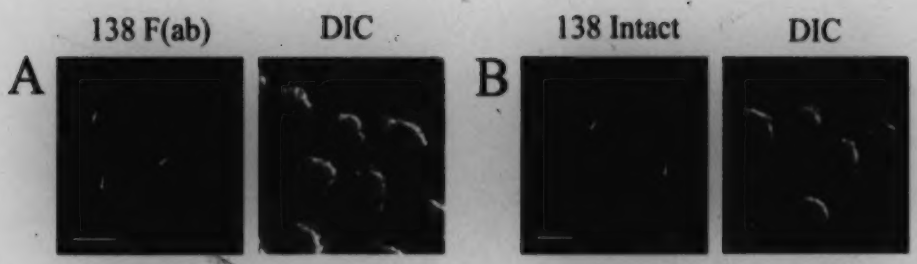

Figure 9. Integrin β1, when ligated with an anti-β1 138 F(ab) fragment or an intact anti-β1 mAb 138 and cross-linked with a goat anti-mouse F(ab)'2-FITC secondary antibody at 37°C, patches and internalizes in U937 cells. Cells were incubated with anti-β1 138 F(ab) fragments (A) or anti-β1 intact mAb 138 (B) at 4°C, then cross-linked with goat anti-mouse F(ab)'2-FITC conjugated fragments at 37°C. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panel A shows that β1 integrin ligated and cross-linked with antibodies lacking Fc regions still internalizes, indicating that integrin endocytosis is not a result of Fc-receptor activation. For comparison purposes, Panel B shows β1 integrin ligated with an intact 138 mAb, containing a Fc region, internalizes at similiar rates as the 138 F(ab) fragments. β1 integrin, green. Bars, 10 μm. Arrows indicate points of β1 integrin internalization.

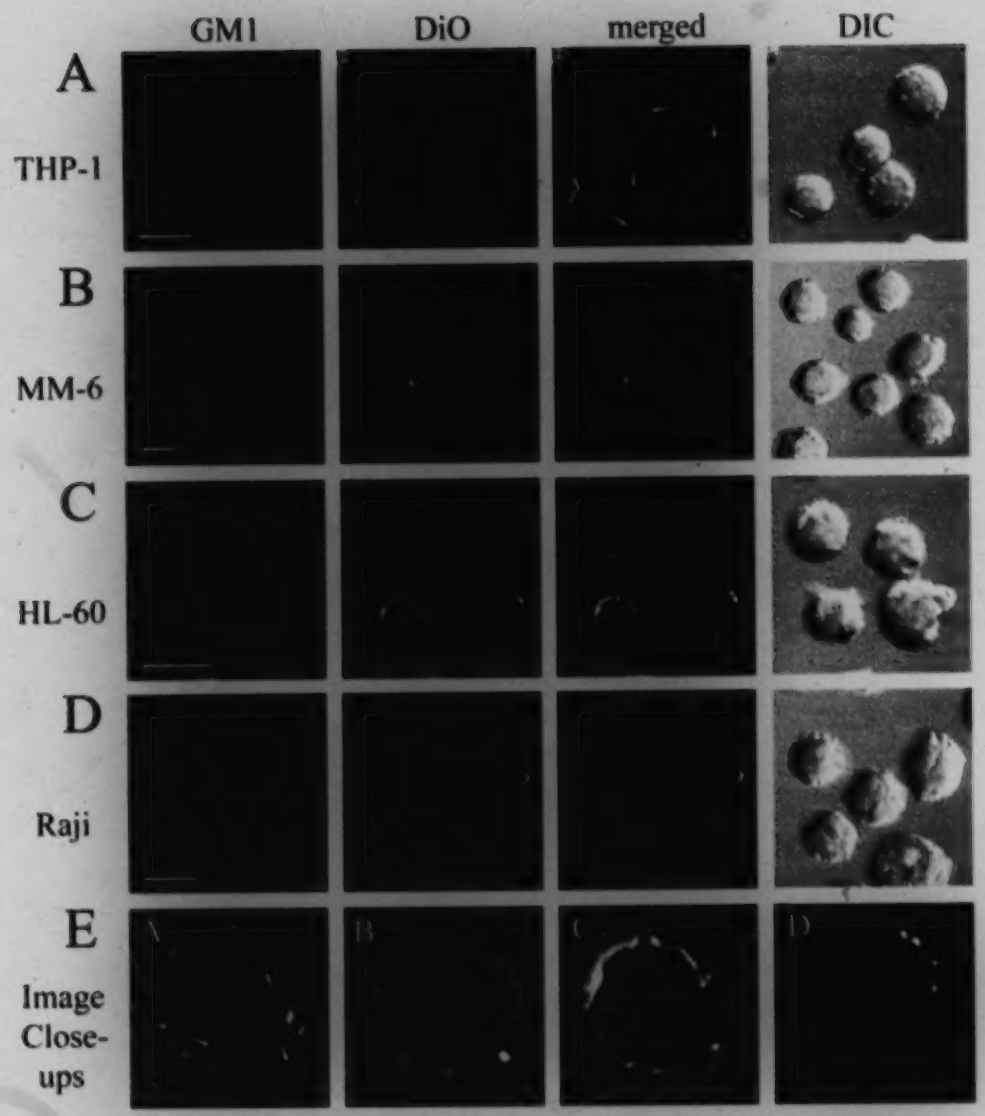

Figure 10. GM1 cross-linked endocytosis is a property of monocytes. Cells were incubated with CtxB-Alexa 594 at 4˚C, then cross-linked with rabbit anti-CtxB at 37˚C in various cell lines including THP-1 (monocytes), MonoMac-6 (mature monocytes), HL-60 (neutrophilic promyelocytes) and Raji (B-lymphocytes). Cells were then stained with fast DiO to label the plasma membrane green. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Top panel (A) shows engaged GM1 undergoes internalization in THP-1 cells similarly to U937 cells. Panels B, C and D show engaged GM1 remains bound to the cell membrane and does not internalize in the remaining cell lines, indicating lipid raft-mediated endocytosis is a property of monocytes. Panel E shows image close-ups of boxed cells from each panel (A-D). GM1, red; DiO, green. Bars, 10 μm. Arrows indicate points of GM1 internalization.

mature cells [88]. To investigate the kinetics of GM1 internalization in THP-1 cells we cross-linked GM1 at 4°C. Cell were than incubated at 37°C for various intervals (0, 10, 20 and 60 min) and samples removed for fixation. Figure 11A shows GM1 localized to the cell membrane at time 0 min, begins to internalize at 10 min (Figure 11B) and endocytosis increases in proportion to the length of incubation (Figure 11C-11D). The endocytic response to clustering α4β1 integrin in the cell lines closely approximates the response to clustering GM1 as would be expected if the integrin is internalized via lipid rafts. α4β1 integrin readily internalizes in THP1 (Figure 12A) but not in MonoMac-6 (Figure 12B) or Raji (Figure 12D). However, in HL-60 cells α4β1 internalizes even though GM1 does not (Figure 12C) indicating that α4β1 endocytosis in HL-60 cells occurs in a pathway that does not require lipid rafts. Both Figures 10E and 12E represent close-ups of boxed cells from Figures 10A to 10D or from 12A to 12D, respectively to help demonstrate the differences of GM1 and integrin internalization observed between the different cell types.

Section 3.7 Integrin internalization via lipid rafts is a property of monocytes

Since we found that α4β1 integrin internalizes through a lipid raft-dependent pathway in U937 cells, we asked whether the integrin would also internalize through association with lipid rafts in THP-1 and HL-60 cells. α4β1 was cross-linked with a secondary antibody at 4°C and GM1 was labeled with CtxB-Alexa 594 at 4°C to identify lipid rafts. The cells were subsequently incubated at 37°C to promote endocytosis. Cross-linked α4β1 integrin

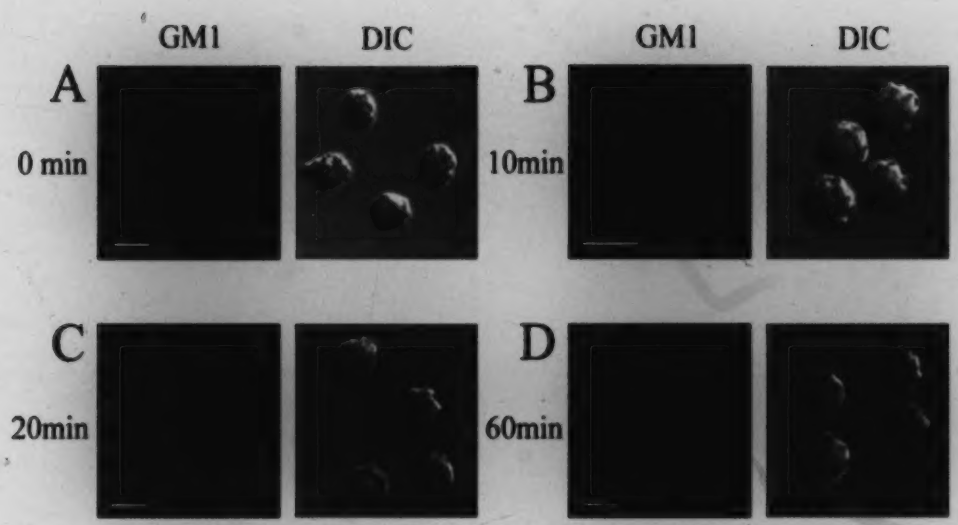

Figure 11. Time scale of GM1 internalization in THP-1 monocytic cells. Cells were incubated with CtxB-Alexa 594 at 4°C, then cross-linked with rabbit anti-CtxB at 4°C. Cells were then incubated for various time points (0, 10, 20 and 60 min) at 37°C. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panel A shows time at 0 min, GM1 is located on the cell membrane and has not undergone any internalization. Panels B through D show GM1 undergoes internalization rapidly (within 10 min) and continues to internalize throughout the hour. GM1, red. Bars, 10 μm.

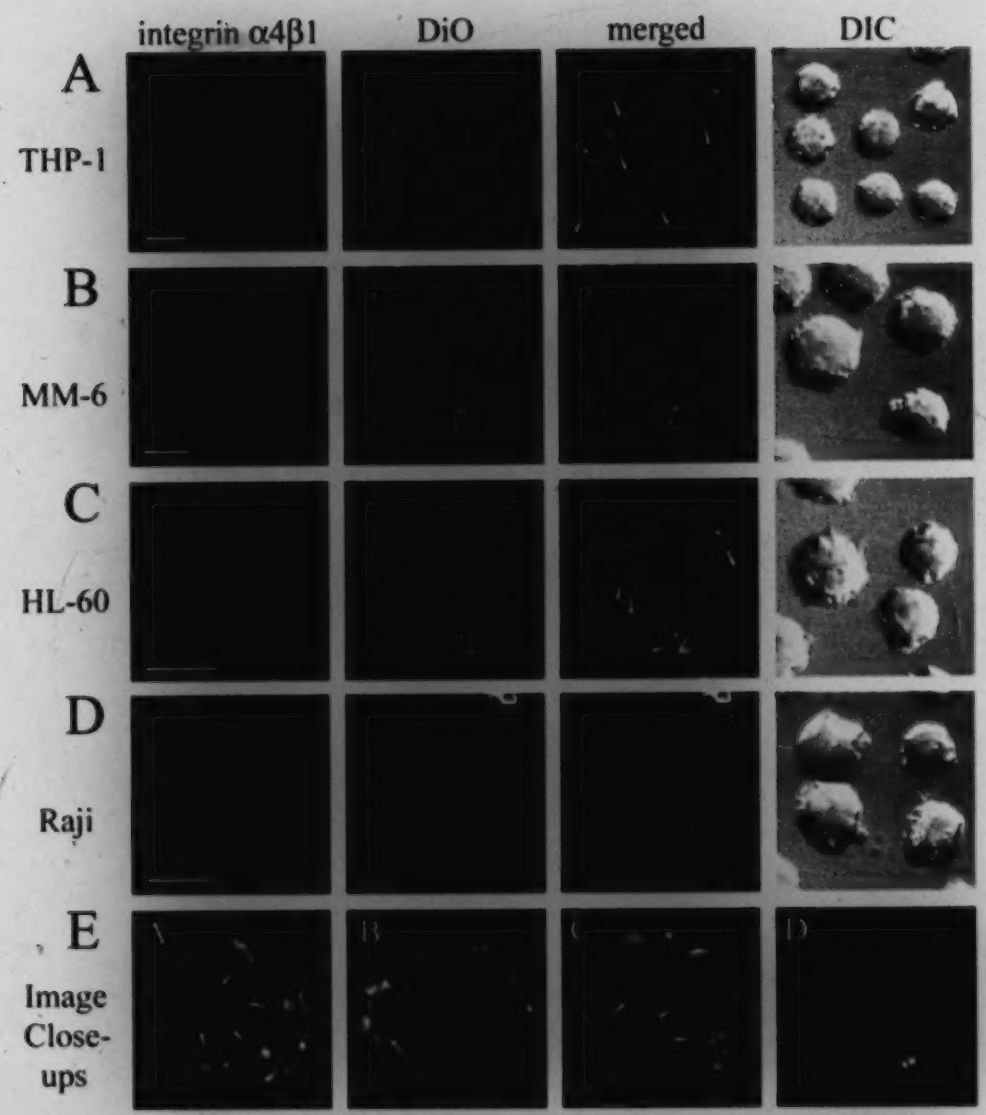

Figure 12. α4β1 integrin cross-linked endocytosis is a property of monocytes and myelocytes. Cells were incubated with anti-α4β1 mAb 44H.6 at 4°C, then cross-linked with goat anti-mouse Ab-Alexa 546 at 37°C in various cell lines including THP-1 (monocytes), MonoMac-6 (mature monocytes), HL-60 (neutrophilic promyelocytes) and Raji (B-lymphocytes). Cells were then stained with fast DiO to label the plasma membrane green. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panels (A & C) show engaged α4β1integin undergoes internalization in THP-1 and HL-60 cells. Panels (B & D) show engaged α4β1 undergoes no internalization in MonoMac-6 or Raji cells, indicating integrin mediated endocytosis is somewhat cell-specific. Panel E shows image close-ups of boxed cells from each panel (A-D). α4β1 integrin, red; DiO, green. Bars, 10 μm. Arrows indicate points of integrin internalization.

internalized with GM1 in THP-1 monocytic cells (Figure 13A), as observed in U937 cells, but not in HL-60 promyelocytic cells (Figure 13B), further indicating that integrin lipid raft-mediated endocytosis is a property of the monocytic lineage. Figure 13C represents close-ups of boxed cells from Figures 13A and 13B to help demonstrate the GM1/integrin co-localization differences observed between the two cell lines.

Section 3.8 GM1 and integrin endocytosis is a clathrin-independent process.

Caveolae and clathrin-coated pits are the two major endocytic pathways utilized for the internalization of membrane proteins. We used RT-PCR analysis to confirm that the U937 cell line is devoid of caveolin-1 protein required for caveolar endocytosis [89] (Figure 14). To determine whether GM1 and the β1 integrins internalize via a clathrin-independent process we treated U937 cells with a hypertonic (0.45 M) sucrose solution reported to inhibit clathrin-dependent endocytosis [81] (Figure 15). As a positive control cells were labeled with transferrin-Alexa 546 at 37°C (Figure 15A and 15B), a molecule known to undergo clathrin-dependent endocytosis [81]. GM1 (Figure 15C), α4β1 (Figure 15D), and α5β1 (Figure 15E) were cross-linked at 37°C with fluorescent secondary antibodies as described in the materials and methods in the 0.45 M sucrose treated cells. Fast-DiO was used to label the plasma membrane. We found that 0.45 M sucrose treatment did indeed inhibit transferrin internalization (Figure 15B) since transferrin internalized well in the isotonic solution (Figure 15A). However, the high sucrose treatment did not affect endocytosis of GM1 or of the integrins indicating that

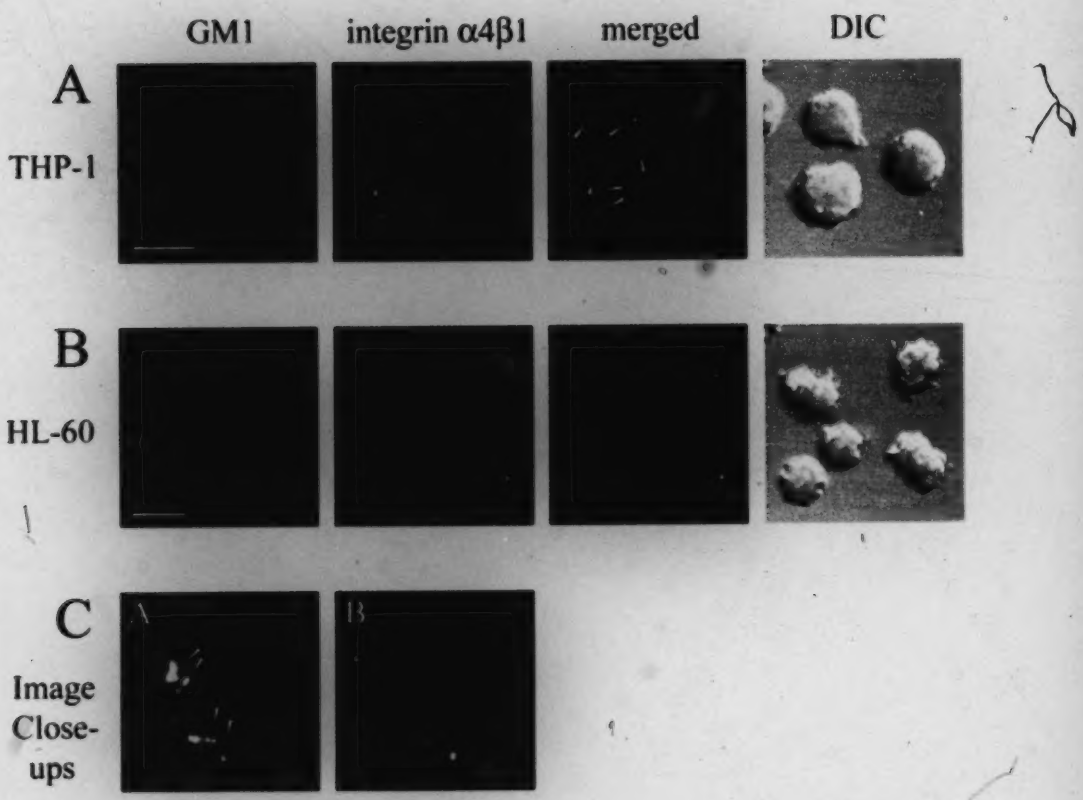

Figure 13. Cross-linked α4β1 integrin co-internalizes with ligated GM1 in THP-1 monocytic cells, but not in HL-60 neutrophilic promyelocytic cells indicating that α4β1 lipid raft-mediated endocytosis is monocyte-cell specific. Cells were incubated with anti-α4β1 mAb 44H.6 at 4˚C, then cross-linked with goat anti-mouse Ab-Alexa 488 at 4˚C. Subsequently, the cells were labeled with CtxB Alexa-594 Ab at 4˚C. Cells were incubated at 37˚C then placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panels (A & B) show cross-linked α4β1 integrin co-internalizes GM1 in THP-1 cells but not in HL-60 cells. Panel C shows image close-ups of boxed cells from each panel (A & B). α4β1 integrin, green; GM1, red. Bars, 10 μm. Arrows indicate points of GM1/integrin co-localization.

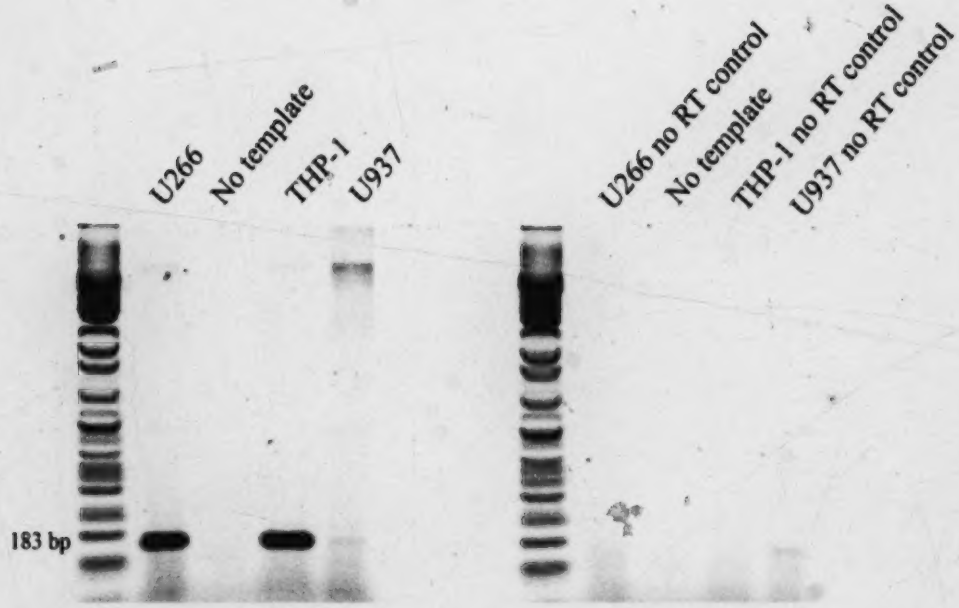

Figure 14. RT-PCR analysis of caveolin-1 transcripts in U266, THP-1, and U937 cells correspond to the expected product size of 183 bp. Equal amounts of RNA were reverse-transcribed to generate cDNA, which was subjected to caveolin-1 specific PCR amplification using paired primers as described in methods and materials. Positive caveolin-1 expression was detected in the caveolin-1 expressing U266 cell line (positive control) and in THP-1 cells. No caveolin-1 expression was detectable in the U937 cell line. No genomic DNA contamination was detected in the -RT negative controls. β2 microglobulin controls were positive for all cDNA samples (not shown) indicating the presence of cDNA in each sample.

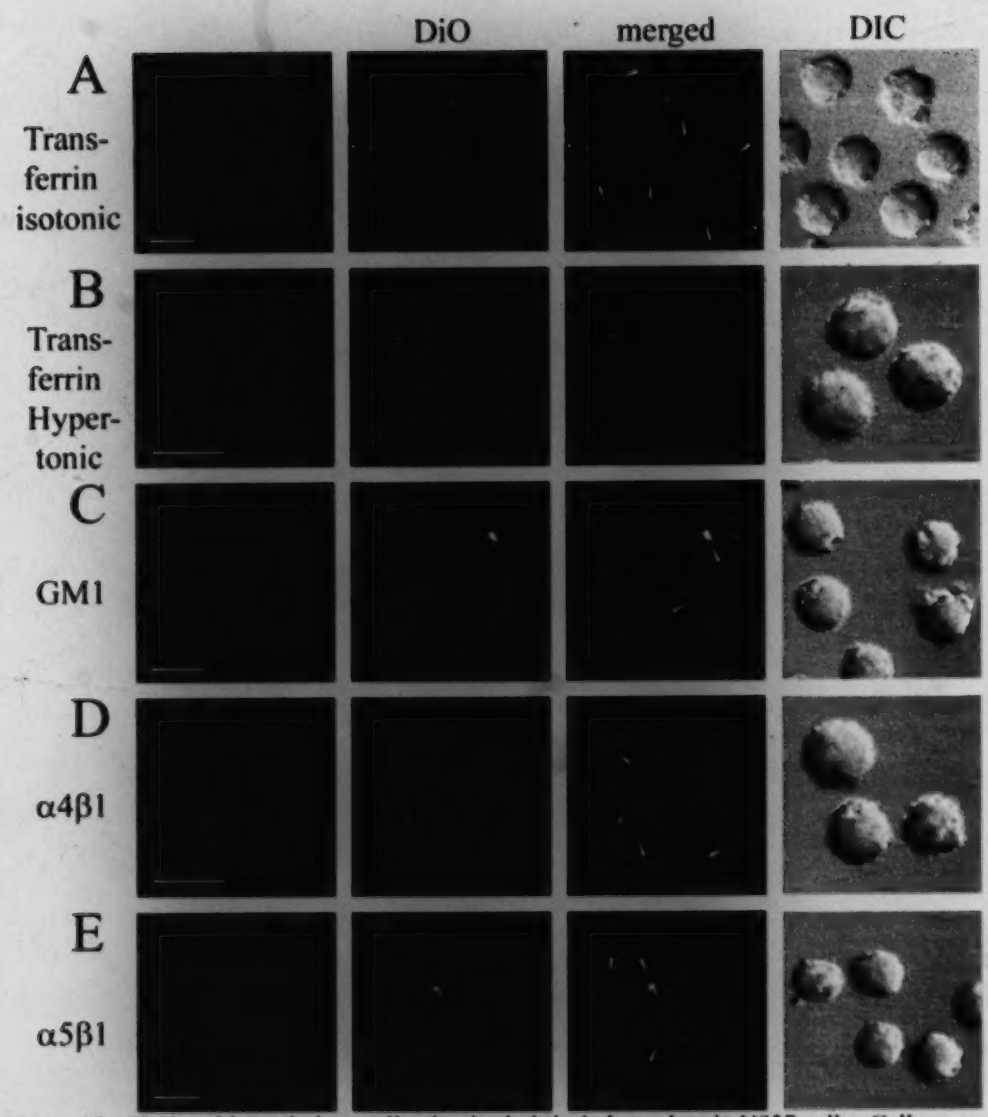

Figure 15. GM1 and integrin internalization is clathrin-independent in U937 cells. Cells were incubated with CtxB-biotin (B), anti-α4β1 mAb 44H.6 (C), or anti-α5β1 mAb P1D6 (D) at 4°C, then cross-linked with rabbit anti-CtxB Ab (B) or goat anti-mouse Ab-Alexa 546 (C & D) at 37°C. During the 37°C incubation, cells were treated with 0.45 M sucrose hypertonic solution. As a control, cells in hypertonic and isotonic solutions were incubated with transferrin Alexa-546 at 37°C (A), known to undergo clathrin-dependent endocytosis. Cells were then stained with fast DiO. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panel A shows transferrin internalizes in the isotonic solution (A) but not in the hypertonic treated U937 cells (B). Panels (C, D & E) show cross-linked GM1 and integrins still undergo internalization in high sucrose hypertonic treated U937 cells. GM1, integrins and transferrin red; DiO, green. Bars, 10 μm. Arrows indicate points of internalization.

endocytosis of both GM1 and integrins is clathrin-independent.

Section 3.9 Cross-linked fibronectin internalizes in monocytic cells.

Fibronectin, a component of the extracellular matrix, is a ligand for both α4β1 and α5β1 integrin. To investigate the functional relevance of rapid integrin internalization in monocytes we asked whether cross-linking membrane-associated fibronectin could induce lipid raft-mediated endocytosis. To accomplish this we biotinylated intact plasma fibronectin and incubated both U937 and THP1 monocytic cells with the biotin conjugated fibronectin at 4˚C. Following removal of unbound fibronectin by washing, we cross-linked the ligated fibronectin with streptavidin-Alexa 546 at 37˚C. By using a biotin/streptavidin combination to cross-link fibronectin we avoided possible activation of Fc receptor endocytosis. Again, we labeled the plasma membrane with DiO. In this experiment, DiO did not only label the plasma membrane but also internal membranes in the cytoplasm. We observed that, in both U937 and THP1 monocytic cells, soluble fibronectin bound to the plasma membrane, that following cross-linking, clustered and internalized (Figure 16A and 16B). Therefore antibody-induced integrin internalization may mimic occupation by clustered fibronectin. A 4°C control was also performed in which cells were labled with fibronectin and streptavidin at 4°C. Under these conditions, we observed only weak staining of fibronectin suggesting that cells must be incubated at 37°C to bind fibronectin avidly.

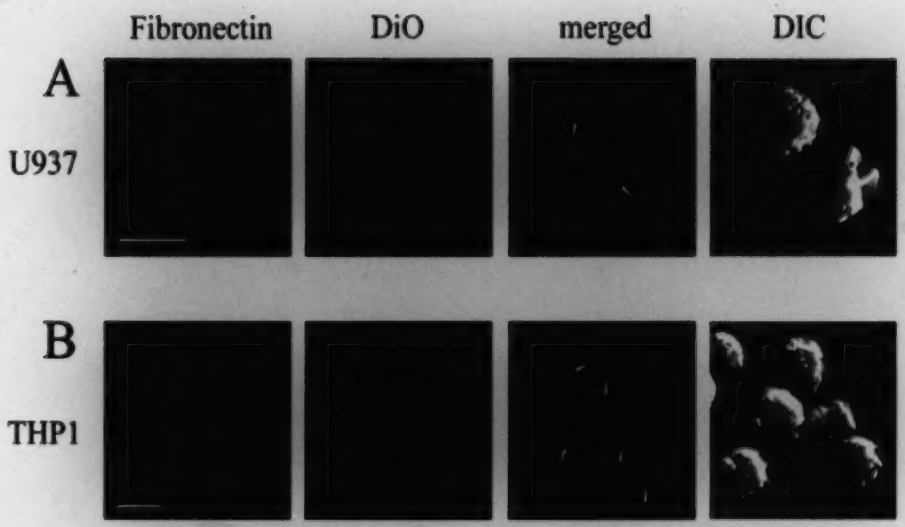

Figure 16. Cross-linking biotin conjugated fibronectin with streptavidin Alexa-546 internalizes in both U937 and THP-1 cells. Cells were incubated with fibronectin-biotin conjugated (A & B) at 4°C, then cross-linked with streptavidin-Alexa 546 at 37°C (A & B). Cells were then stained with fast DiO to label the plasma membrane green. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panels (A & B) show fibronectin-biotin binds to monocytic cells and upon cross-linking with streptavidin Alexa-546 at 37°C internalizes. Fibronectin, red; DiO, green. Bars, 10 μm. Arrows indicate points of fibronectin internalization.

Section 3.10 What happens to the internalized integrins?

To investigate the route of lipid-raft mediated endocytosis and determine whether integrins recycle back to the plasma membrane or traffic to lysosomes where ligands might be degraded we looked for evidence of co-localization of internalized GM1 and integrins with the endosomal marker transferrin and the lysosomal marker lysotracker. Cells were incubated with CtxB-Alexa 488, the anti-α4β1 mAb 44H.6 or the anti-α5β1 mAb P1D6 at 4˚C, and cross-linked with rabbit anti-CtxB antiserum or with goat anti-mouse Ab-Alexa 488 at 4˚C. Cells were then incubated with lysotracker-DND 99 or with transferrin-Alexa 546 at 37˚C for up to 2 hours. We observed that neither GM1 nor the integrins co-clustered extensively with transferrin or lysotracker indicating that the endocytosis of GM1, α4β1 or α5β1 does not occur through association with recycling endosomes (Figure 17A, 17B and 17C) or trafficking to lysosomes (Figure 18A, 18B and 18C). Thus lipid raft-mediated endocytosis may direct integrins into distinct and possibly unique trafficking pathways.

Section 3.11 Development of methods to quantify integrin and GM1 internalization

Having established that integrins internalize through a lipid raft-mediated mechanism we wished to investigate the cytoskeletal and signaling pathways involved through use of specific inhibitors. However, in order to compare the effects of inhibitors it was necessary to derive a method for quantifying the endocytic process. After several

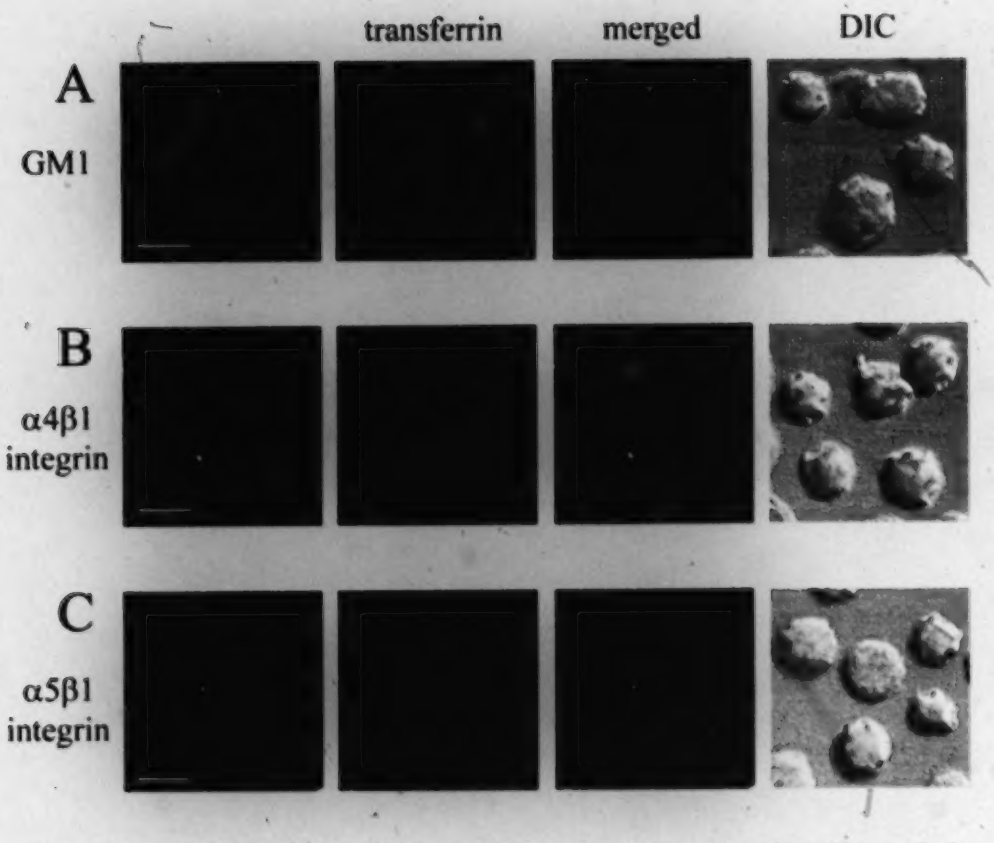

Figure 17. Cross-linked GM1, α4β1 and α5β1 integrins do not co-localize with transferrin. Cells were incubated with CtxB-Alexa 488 (A), anti-α4β1 mAb 44H.6 (B) or anti-α5β1 mAb P1D6 (C) at 4˚C, then cross-linked with rabbit anti-CtxB Ab (A) or goat anti-mouse Ab-Alexa 488 (B & C) at 4˚C. The cells were then incubated with transferrin Alexa-546 at 37˚C. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Top panel (A) shows cross-linked GM1 does not co-cluster transferrin. Bottom panels show cross-linked α4β1 (B) and α5β1 (C) integrins also do not co-cluster transferrin, concluding that internalized GM1 or integrins do not associate with endosomes. GM1, α4β1 and α5β1 integrins, green; transferrin, red. Bars, 10 μm.

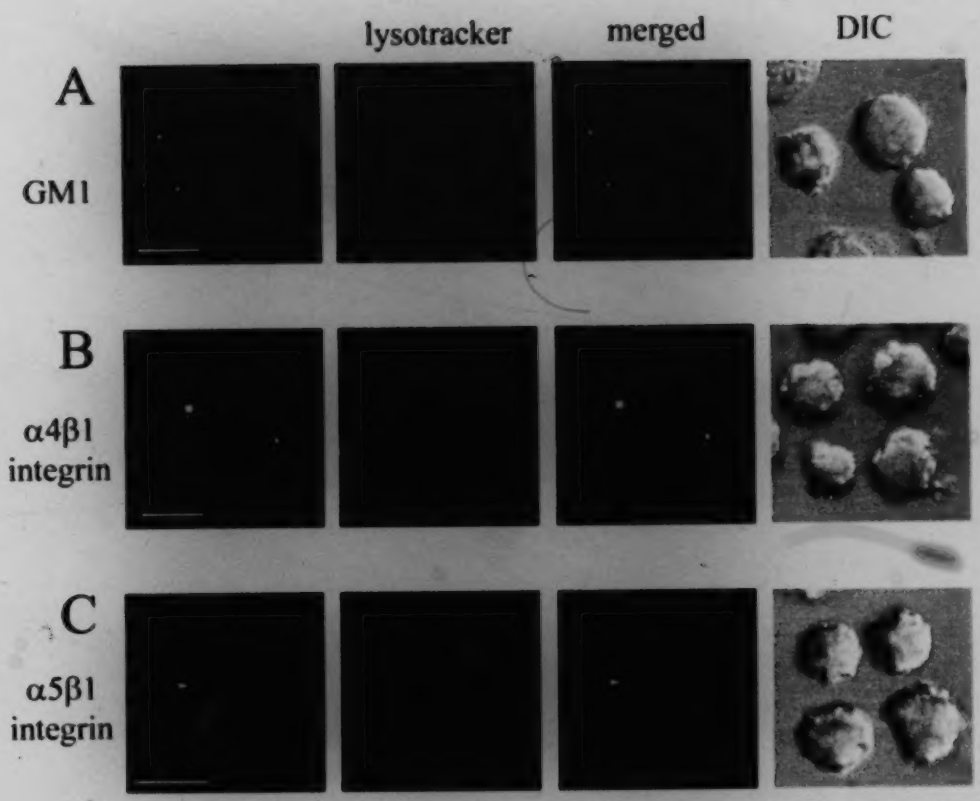

Figure 18. Cross-linked GM1, α4β1 and α5β1 integrins do not co-localize with lysosomes. Cells were incubated with CtxB-Alexa 488 (A), anti-α4β1 mAb 44H.6 (B) or anti- α5β1 mAb P1D6 (C) at 4˚C, then cross-linked with rabbit anti-CtxB Ab (A) or goat anti-mouse Ab-Alexa 488 (B & C) at 4˚C. The cells were then incubated with lysotracker Alexa-546 at 37˚C. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Top panel (A) shows cross-linked GM1 does not co-cluster lysotracker. Bottom panels show cross-linked α4β1 (B) and α5β1 (C) integrins only partially co-cluster lysotracker, concluding that internalized GM1 or integrins generally do not undergo degradation. GM1, α4β1 and α5β1 integrins, green; lysotracker, red. Bars, 10 μm.

unsuccessful attempts to develop methods to displace ligands from the cell surface in order to measure internalized ligand, we realized that it would be simpler and more effective to quantify the ligand remaining on the surface after the incubation period through labeling with a tertiary fluorescent antibody. We first incubated the cells with the anti-α4β1 mAb 44H.6 or with CtxB-Alexa 594 at 4˚C for 45 min, and then cross-linked with goat anti-mouse Ab-Alexa 546 or rabbit anti-CtxB at 37˚C for 60 min. To investigate the effect of inhibiting signaling pathways we added various inhibitors just prior to the 37˚C incubation period. However, to investigate the role of cholesterol U937 cells were treated with 15 mM MβCD for 15 min at 37˚C prior to integrin/GM1 cross-linking at 37˚C. To quantify the amount of α4β1 or GM1 remaining on the cell surface, cells were incubated with the tertiary Ab donkey anti-goat Cy5 (for α4β1) or with goat anti-rabbit Cy5 (for GM1) for 45 min at 4˚C. Since the secondary Ab would excite at one wavelength, whereas the tertiary Ab would excite at a different wavelength we could therefore differentiate between the two antibodies. To quantify internalization using confocal microscopy we took an optimal number of Z-stack optical sections from 20 individual cells for each sample set. Using Imaris co-localization software, we measured the intensity of each pixel for both wavelengths to obtain co-localization channel correlations from the data set volume for all 20 of the 3-dimensionally reconstructed cells. The correlations were then averaged and graphed. High correlations (close to 1) indicated little to no internalization, whereas low correlation values (close to 0) indicated high amounts of internalization.

Figure 19 is a representative example of the images used to quantitate internalization of α4β1 and GM1. Figures 19A and 19B contain cross sections through the equator of cells illustrating the effect of cross-linking α4β1 and GM1 respectively at 4°C and detecting membrane bound ligand with tertiary antibodies. By maintaining the temperature at 4°C we inhibit internalization and therefore observe a strong correlation between the co-localization of the secondary and tertiary antibodies on the plasma membrane. However, upon incubating the secondary antibody at 37°C we induce internalization of α4β1 integrin and GM1. Since the tertiary antibody is incubated at 4°C, it binds to the remaining membrane-bound antibody. Therefore, we observe a significant decrease in co-localization of the secondary and the tertiary antibodies (Figures 19C and 19D).

We first quantitated the amount of integrin and GM1 internalization inhibited by MβCD treatment. MβCD treatment completely abolished endocytosis of both integrins and GM1 (Figure 20A and 20B). In this case, the amount of internalization observed in the MβCD treated cells was equivalent to the amount of internalization in the 4°C cross-linked control cells (Figure 21 and 22). This result provides quantitative support for our observation that integrin and GM1 internalization requires membrane cholesterol.

We then proceeded to examine whether an intact cytoskeleton is required for integrin and GM1 internalization. We treated the U937 cells with cytochalasin D to disrupt F-actin, and with nocodazole to inhibit microtubule polymerization. We discovered that cytochalasin D inhibited both integrin and GM1 internalization but nocodazole treatment had no inhibitory effect, indicating that F-actin but not microtubule polymerization was

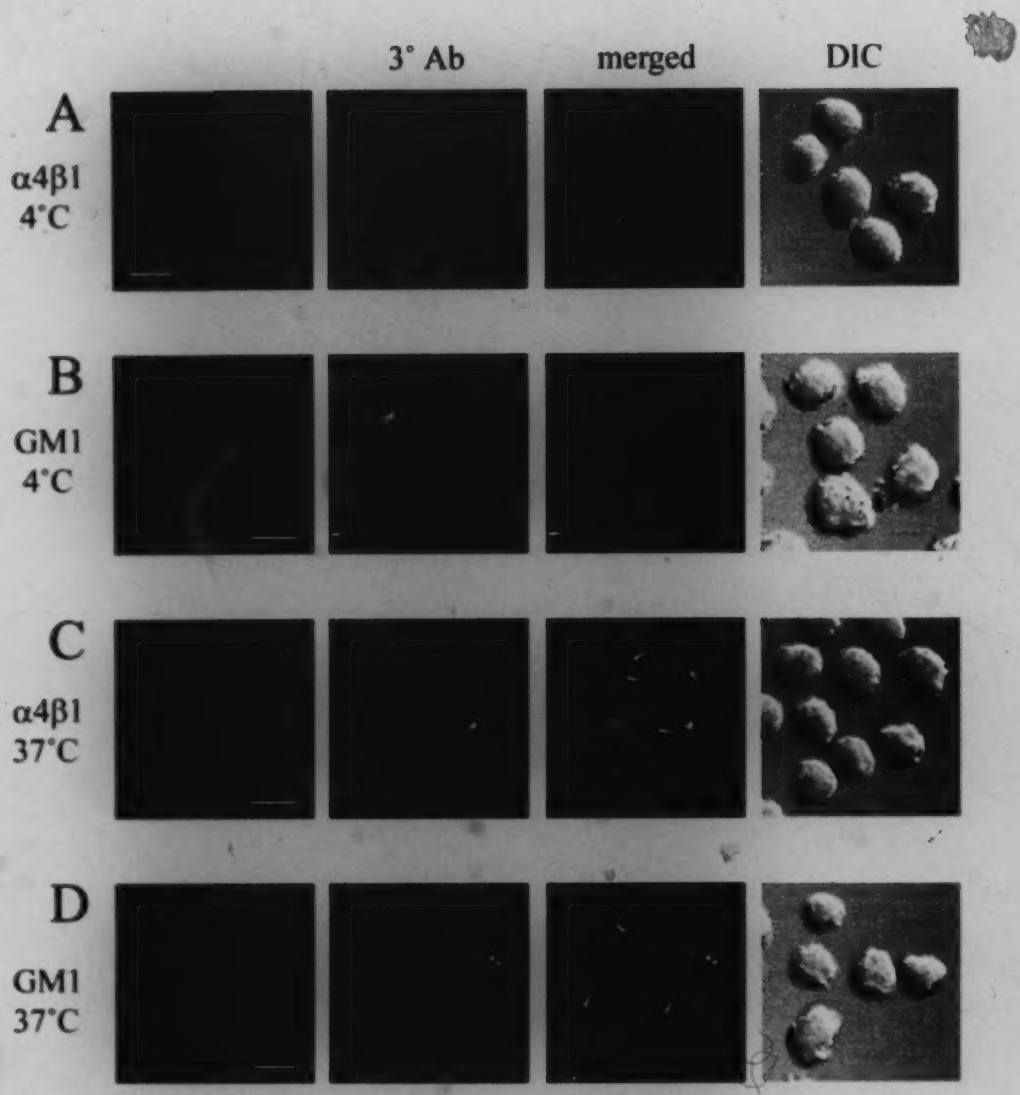

Figure 19. Quantitation of integrin α4β1 and GM1 internalization in U937 cells compared to untreated U937 cells. Cells were incubated with anti-α4β1 mAb 44H.6 (A & C) or CtxB Alexa-594 (B & D) at 4°C, then cross-linked with goat anti-mouse Ab-Alexa 546 at 4°C (A) or at 37°C (C) or rabbit anti-CtxB at 4°C (B) or at 37°C (D). Cells were then incubated with tertiary Ab donkey anti-goat Cy5 (A & C) or goat anti-rabbit Cy 5 (B & D) at 4°C. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Top panels (A & B) show α4β1 and GM1 co-localize to the tertiary antibody on the plasma membrane when cross-linked at 4°C. Bottom panels (C & D) show α4β1 and GM1 when cross-linked at 37°C, internalize and only partially co-localize with the tertiary antibody on the cell membrane. α4β1 integrin and GM1, red; tertiary Ab, green. Bars, 10 μm. Arrows indicate points of GM1 and integrin internalization.

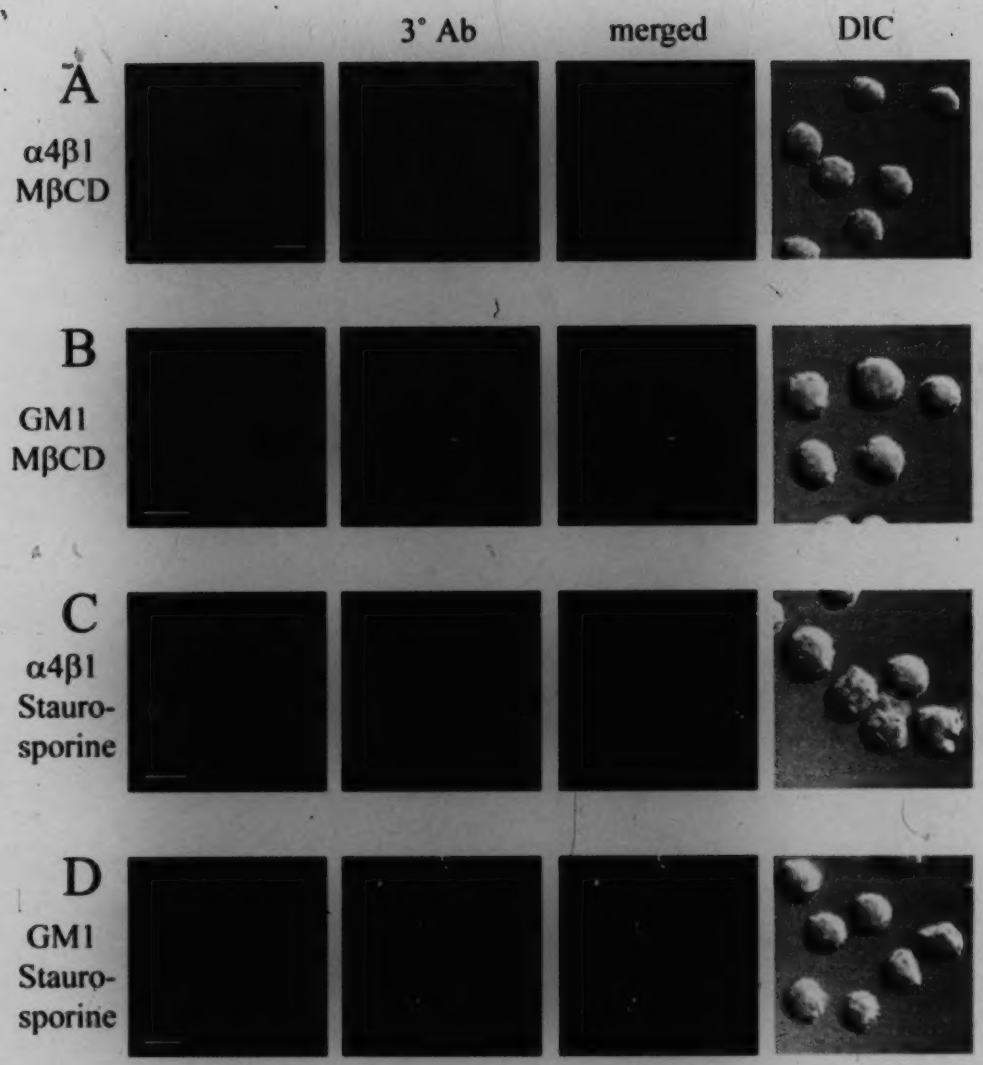

Figure 20. Quantitation of integrin α4β1 and GM1 internalization in U937 cells treated with MβCD or staurosporine. Cells were incubated with anti-α4β1 mAb 44H.6 (A & C) or CtxB-Alexa 594 (B & D) at 4°C, then cross-linked with goat anti-mouse Ab-Alexa 546 at 37°C (A &C) or rabbit anti-CtxB at 37°C (B & D). Cells were then incubated with tertiary Ab donkey anti-goat Cy5 (A & C) or goat anti- rabbit Cy 5 (B & D) at 4°C. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Top panels (A & B) show α4β1 and GM1 co-localize to the tertiary antibody on the plasma membrane when U937 cells are treated with MβCD. Bottom panels (C & D) show α4β1 and GM1 co-localize to the tertiary antibody on the plasma membrane when U937 cells are treated with staurosporine. α4β1 integrin and GM1, red; tertiary Ab, green. Bars, 10 μm.

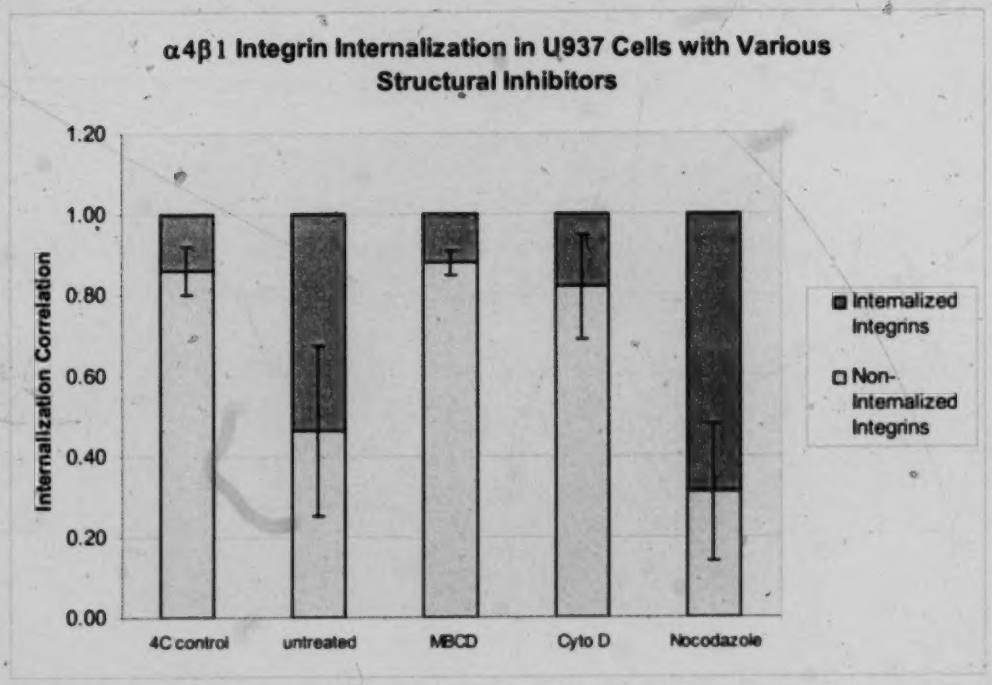

Figure 21. Quantitation of α4β1 endocytosis with inhibitors of compartmentalization and cytoskeleton organization. Quantitation was completed using a tertiary antibody system. We first incubated the cells with anti-α4β1 mAb 44H.6 at 4˚C for 45min, then cross-linked with goat anti-mouse Ab-Alexa 546 at 37˚C for 60 min. During this 37˚C incubation cells were simultaneously treated with the various inhibitors. Except for MβCD treatment, the U937 cells were treated with MβCD for 15 min at 37˚C prior to integrin cross-linking. Cells were then incubated with tertiary Ab donkey anti-goat Cy5 for 45 min at 4˚C. Internalization quantitation was completed by taking Z-stack optical sections of 20 individual cells for each sample set. Using Imaris co-localization software, we determined co-localization channel correlations for the data set volume of all 20 cells in each data set. The correlations were then averaged and graphed. Error bars represent +/- 1 standard deviation about the mean.

MβCD and cytochalasin D treatment inhibited integrin endocytosis suggesting that intact lipid rafts and F-actin are required for integrin internalization. Nocodazole treatment had no inhibitory effects, indicating that microtubule polymerization is not required for integrin endocytosis.

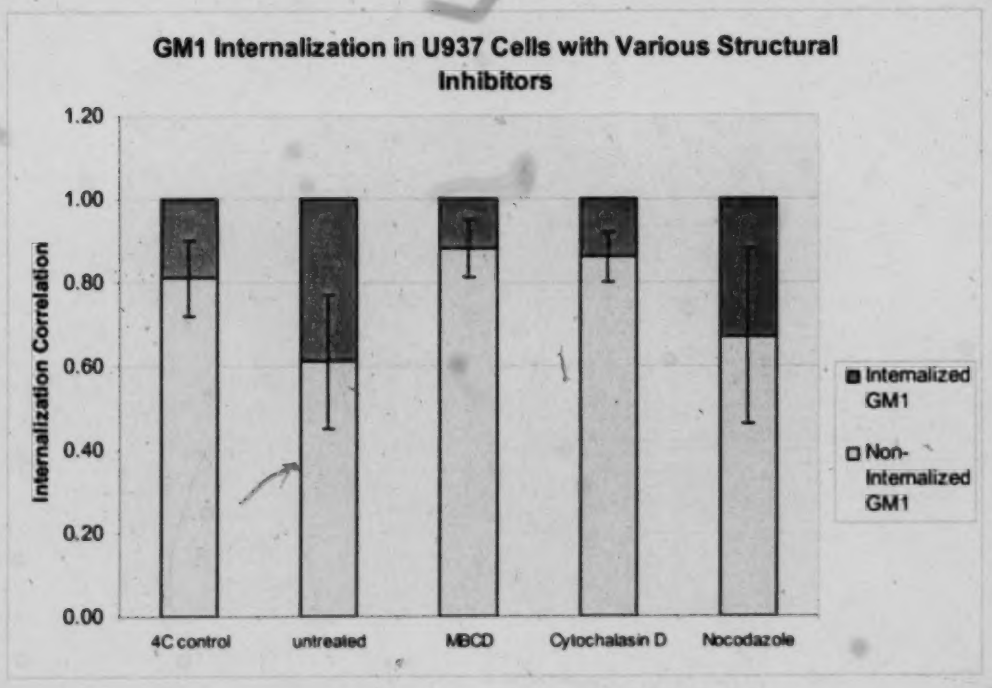

Figure 22. Quantitation of GM1 endocytosis with inhibitors of compartmentalization and cytoskeleton organization. Quantitation was completed using a tertiary antibody system. We first incubated the cells with CtxB-Alexa 594 (GM1) at 4˚C for 45min, then cross-linked with rabbit anti-CtxB at 37˚C for 60 min. During this 37˚C incubation cells were simultaneously treated with the various inhibitors. Except for MβCD treatment, the U937 cells were treated with MβCD for 15 min at 37˚C prior to GM1 cross-linking. Cells were then incubated with tertiary Ab goat anti-rabbit Cy5 for 45 min at 4˚C. Internalization quantitation was completed by taking Z-stack optical sections of 20 individual cells for each sample set. Using Imaris co-localization software, we determined co-localization channel correlations for the data set volume of all 20 cells in each data set. The correlations were then averaged and graphed. Error bars represent +/- 1 standard deviation about the mean.
MβCD and cytochalasin D treatment inhibited GM1 endocytosis suggesting that intact lipid rafts and F-actin are required for GM1 internalization. Nocodazole treatment had no inhibitory effects, indicating that microtubule polymerization is not required for GM1 endocytosis.

required for internalization (Figures 21 and 22). Again, the amount of internalization observed with cytochalasin D treatment was equivalent to the amount of internalization measured in the 4°C cross-linked control cells (Figures 21 and 22). Therefore lipid-raft mediated endocytosis appears to involve actin-dependent cytoskeletal reorganization.

We investigated the participation of signaling pathways involved in integrin and GM1 induced endocytosis by treating the cells with inhibitors of signaling molecules. U937 cells were treated with staurosporine (a broad inhibitor of serine/threonine kinases including protein kinase C), PP1 and PP2 (inhibitors of Src-family tyrosine kinases), wortmannin (a fungal metabolite that inhibits PI-3 kinase), genistein (a broad spectrum protein-tyrosine kinase inhibitor), Bapta AM (to sequester intracellular calcium), and W-7 (an inhibitor of the calcium-binding protein calmodulin). Although most experiments were performed in RPMI-1640, Bapta-AM was incubated in calcium-free PBS. To control for the absence of external cations we performed a PBS control, devoid of Bapta AM, to confirm that the integrins and GM1 could still internalize. Staurosporine inhibited both $\alpha 4\beta 1$ and GM1 internalization (Figures 23 and 24) resulting in good co-localization between the secondary and tertiary antibodies (Figures 20C and 20D). Our finding that $\alpha 4\beta 1$ and GM1 induced endocytosis are both sensitive to inhibitors of F-actin assembly, serine/threonine kinases and to cholesterol depletion is further evidence that they trigger a common endocytosis mechanism.

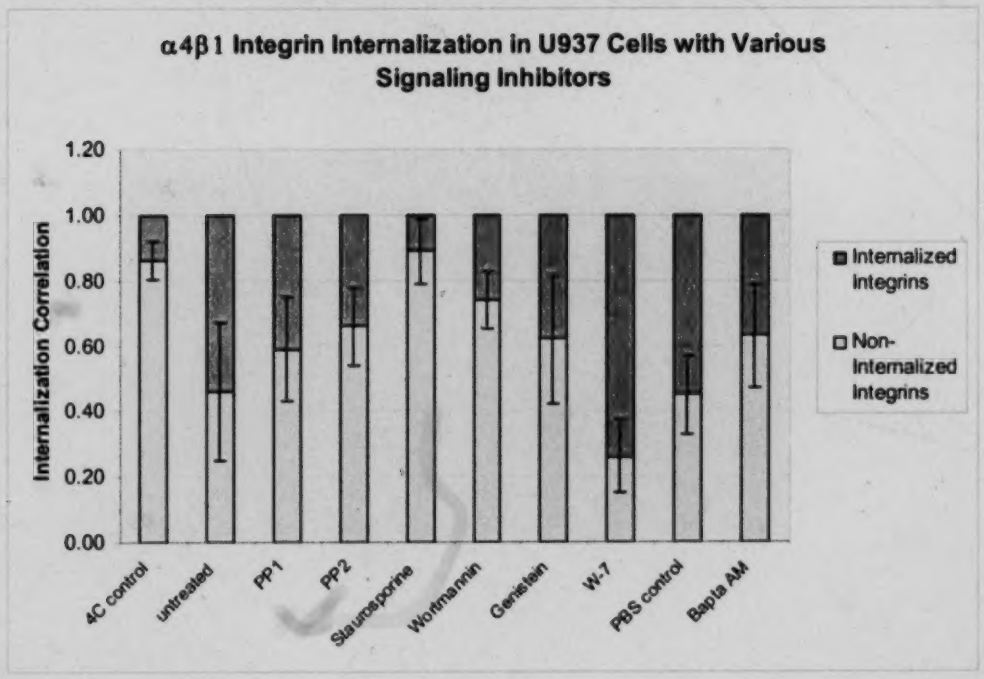

Figure 23. Quantitation of α4β1 endocytosis with various inhibitors of signaling. Quantitation was completed using a tertiary antibody system. We first incubated the cells with anti-α4β1 mAb 44H.6 at 4˚C for 45min, then cross-linked with goat anti-mouse Ab-Alexa at 37˚C for 60 min. During this 37˚C incubation cells were simultaneously treated with the various inhibitors. Cells were then incubated with tertiary Ab donkey anti-goat Cy5 for 45 min at 4˚C. Internalization quantitation was completed by taking Z-stack optical sections of 20 individual cells for each sample set. Using Imaris co-localization software, we determined co-localization channel correlations for the data set volume of all 20 cells in each data set. The correlations were then averaged and graphed. Error bars represent +/- 1 standard deviation about the mean.
U937 cells were treated with either staurosporine, PP1 and PP2 analogs, wortmannin, W-7 or genistein. Lastly, the cells were treated with Bapta AM which sequesters internal calcium. To utilize Bapta AM we needed to switch cell media to PBS from RPMI to avoid replenishment of calcium. Therefore, we performed a PBS control without Bapta AM to make sure the integrins upon cross-linking would still internalize. The data indicates that staurosporine strongly inhibits α4β1 internalization but the rest of the signaling inhibitors only partially inhibit endocytosis. Therefore, we can conclude that α4β1 endocytoses with a mechanism that requires cholesterol, F-actin, and protein kinase C.

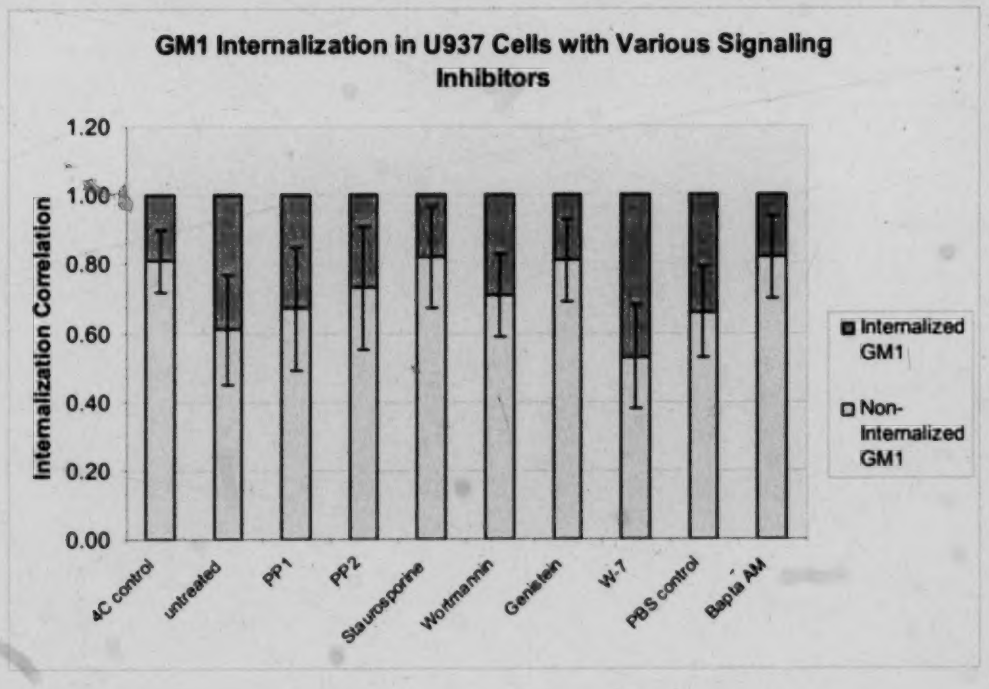

Figure 24. Quantitation of GM1 endocytosis with various inhibitors of signaling. Quantitation was completed using a tertiary antibody system. We first incubated the cells with CtxB-Alexa 594 (GM1) at 4˚C for 45min, then cross-linked with rabbit anti-CtxB at 37˚C for 60 min. During this 37˚C incubation cells were simultaneously treated with the various inhibitors. Cells were then incubated with tertiary Ab goat anti-rabbit Cy5 for 45 min at 4˚C. Internalization quantitation was completed by taking Z-stack optical sections of 20 individual cells for each sample set. Using Imaris co-localization software, we determined co-localization channel correlations for the data set volume of all 20 cells in each data set. The correlations were then averaged and graphed Error bars represent +/- 1 standard deviation about the mean.
U937 cells were treated with either, staurosporine, PP1 and PP2 analogs, wortmannin, W-7 or genistein. Lastly, the cells were treated with Bapta AM which sequesters internal calcium. To utilize Bapta AM we needed to switch cell media to PBS from RPMI to avoid replenishment of calcium. Therefore, we performed a PBS control without Bapta AM to make sure GM1 upon cross-linking would still internalize. The data indicates that staurosporine, genistein and Bapta AM strongly inhibits GM1 internalization but the rest of the signaling inhibitors only partially inhibit endocytosis. Therefore, we can conclude that GM1 endocytosis requires cholesterol, F-actin, protein kinase C (similarly to $\alpha 4\beta 1$ integrin), internal calcium and protein tyrosine kinase activity.

Section 3.12 Chapter summary

Using confocal microscopy we demonstrate that cross-linking α4β1 and α5β1 integrins in the monocytic cell lines THP-1 and U937 at 37°C induces internalization of the integrins within vesicles containing GM1, a constitutive marker for lipid rafts, and that internalization is cholesterol-dependent indicative of a lipid raft-mediated mechanism. Since cross-linking GM1 triggers endocytosis of GM1 but not β1 integrin, lipid raft internalization does not require the participation of β1 integrins. However, β1 integrins co-cluster on the cell surface with GM1 indicating that the integrins have an affinity for lipid rafts, but require occupation of the integrin in order to internalize. We demonstrate that lipid raft-mediated endocytosis is specific to promonocytes and proceeds independently of Fc receptor activation, or the involvement of caveole or clathrin-coated pits. We also show that cross-linking fibronectin on the cell surface promotes fibronectin internalization suggesting that early monocytic cells clear fibronectin occupied integrins through inclusion in lipid rafts. Internalization of both clustered GM1 and β1 integrins proceeds through a serine/threonine kinase, F-actin and cholesterol-dependent process indicating a common mechanism. Taken together these results indicate that occupation of β1 integrins by soluble ligands triggers a cell-dependent association with lipid rafts that engages a clathrin-independent lipid-raft mediated endocytic mechanism in monocytic cells.

Chapter 4: Results. CD81 Regulation of integrin lipid raft-mediated endocytosis

Section 4.1 Chapter introduction and experimental objectives

From the preceding experiments it appears that the mechanisms by which integrins associate with lipid rafts may critically determine their ability to command raft-based lipid sorting mechanisms involved in endocytosis. One mechanism by which transmembrane proteins may modulate their association with lipid rafts is through protein-protein interaction with proteins that have an affinity for, and could organize lipid raft assemblies. The tetraspanins are molecular facilitators which modulate interactions between tetraspanin-associative proteins such as integrins and the cytoskeleton thereby effecting changes in cellular adhesion and protein trafficking. Exactly how tetraspanins interface lipid rafts and cytoskeletal organization in the context of integrin adhesion is not understood. We hypothesized that since tetraspanins associate with raft-like membrane microdomains [66] and with β1 integrins they may facilitate interactions between β1 integrins and lipid rafts required for cytoskeletal re-organization. Recently ectopic expression of the tetraspanin CD81 in U937 cells was found to enhance adhesion between α4β1 integrin and its ligand VCAM-1 under conditions of shear force [80] suggesting CD81 can modulate α4β1 re-organization of the cytoskeleton. We hypothesized that ectopic expression of CD81 might aid integrin localization to lipid rafts

and therefore facilitate integrin internalization. To study CD81 function, we compared a cloned subline of U937 cells which lack CD81, with U937 cells that are stably transfected with CD81 which were kindly provided by Dr. Shoshana Levy (Stanford University). The level of CD81 expression in U937 cells was investigated by FACS analysis. CD81 transfected U937 cells express CD81 at physiological levels [80].

Section 4.2 Expression of α4β1 and α5β1 integrins is unaltered in CD81 transfected cells

α4β1 and α5β1 are the two major β1 integrins expressed in U937 cells. To confirm that CD81 expression did not alter integrin expression we performed flow cytometry analysis. To accomplish this we labeled the cells first with the anti-β1 mAb 138, and the anti-α4β1 mAb 44H.6 or the anti-α5β1 mAb P1D6 at 4°C and detected the antibodies by secondary labeling with goat anti-mouse Ab conjugated to phycoerythrin (PE) at 4°C. Flow cytometry analysis demonstrated that CD81 expression did not inhibit membrane expression of α4β1 or α5β1 integrin (Figure 25). In support of our results, α4β1 expression was also shown to be unaltered in the same U937/CD81 transfected cell line by FACS analysis [80].

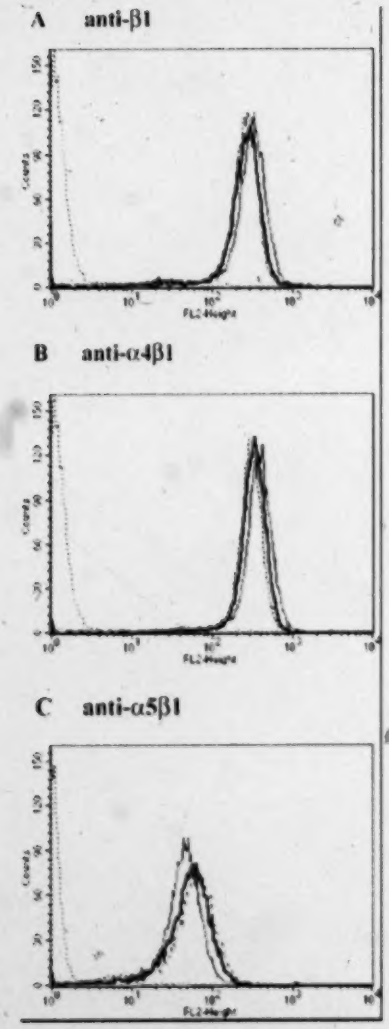

Figure 25. Comparison of α4β1 and α5β1 integrin expression in U937 and U937/CD81 cells by FACS analysis. Cells were labeled with anti-β1 mAb 138 (A), anti-α4β1 mAb 44H.6 (B) or anti-α5β1 mAb P1D6 (C) at 4°C. Cells were then incubated with goat anti-mouse PE at 4°C to fluorescently label the integrins. The cells were then subjected to FACS analysis. The thicker lined curve represents the U937 cell line whereas the thinner lined curve represents the CD81 transfected U937 cell line. Figure indicates that CD81 expression does not inhibit membrane expression of α4β1 or α5β1 integrin.

Section 4.3 Integrin internalization

We have already established that both α4β1 and α5β1 integrin undergo rapid clustering and internalization in U937 cells following antibody cross-linking at 37°C. Additionally, Dr. Shaw had observed that ectopic expression of CD81 in U937 cells increased integrin-dependent adhesion to fibronectin under shear force. Therefore, we anticipated that CD81 expression might alter the rate or quantity of integrin internalization in U937 cells. Cells were incubated with the anti-α4β1 mAb 44H.6 or with anti-α5β1 mAb PID6 at 4°C, and cross-linked with goat anti-mouse Ab-Alexa 546 at 37°C. Cells were placed on poly-L-lysine coated coverslips, fixed, permeabilized, and stained with phalloidin-FITC to label F-actin to differentiate internal from plasma membrane-bound integrins. We stained for F-actin rather than label the membrane with DiO, to observe if CD81 ectopic expression would have an effect on F-actin distribution throughout the experiments. Contrary to expectation, we found that cross-linking either α4β1 (Figure 26A and 26B) or α5β1 (Figure 26C and 26D) integrins at 37°C stimulated integrin patching and internalization equally well in both U937 cells and U937 cells transfected with CD81. Interestingly, we observed that membrane regions associated with high integrin internalization contained higher levels of F-actin indicating involvement of actin polymerization in the endocytic pathway (Figure 26).

In order to quantitate integrin internalization between the two cells lines we utilized the tertiary antibody labeling technique. We cross-linked α4β1 and α5β1 integrins with secondary fluorescent antibodies at 37°C for 60 min as described in the materials and

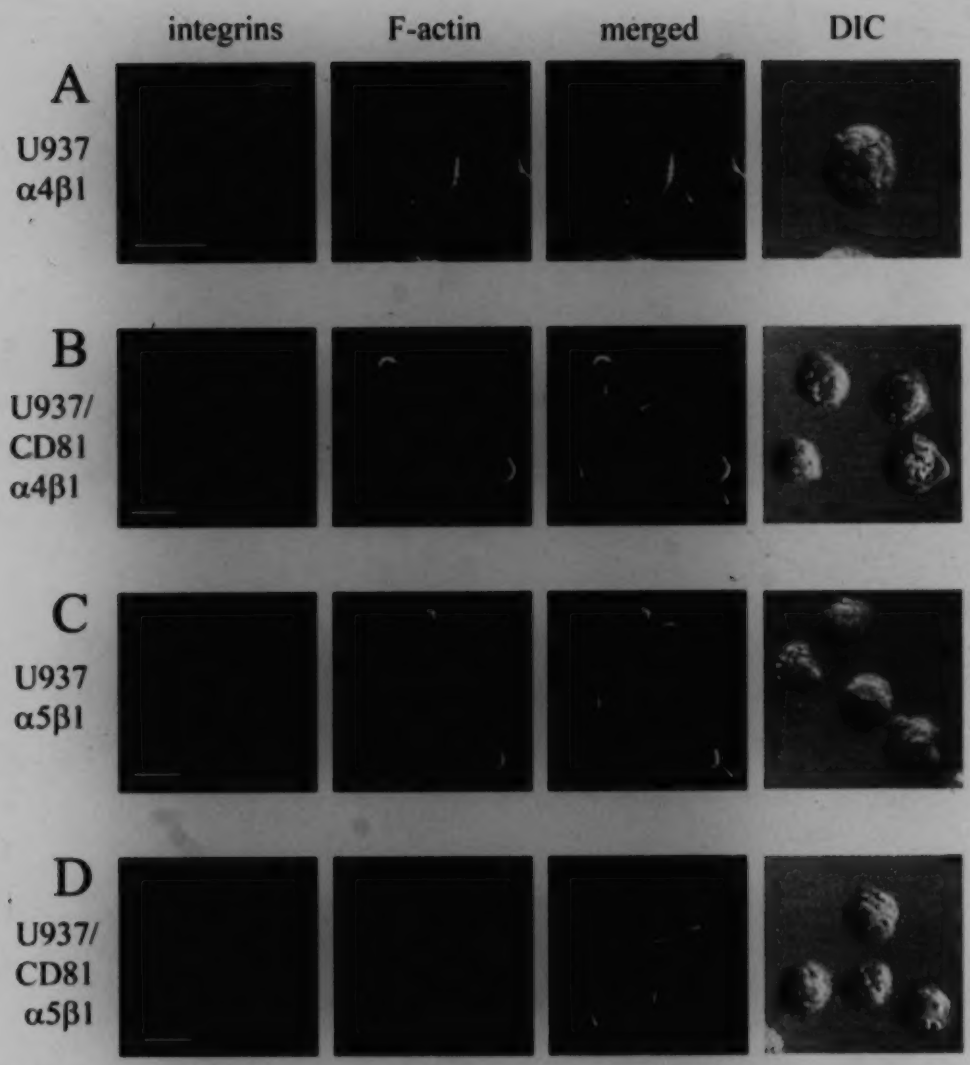

Figure 26. Comparison of α4β1 and α5β1 internalization in both U937 and U937/CD81 cells. Cells were incubated with anti-α4β1 mAb 44H.6 (A & B) or anti-α5β1 mAb P1D6 (C & D) at 4°C, then cross-linked with goat anti-mouse Ab-Alexa 546 at 37°C. Cells were placed on poly-L-lysine, fixed, permeabilized and stained with phalloidin-FITC to label F-actin. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Top panels (A & B) show cross-linked α4β1 integrins patch and internalize in both U937 and U937/CD81 cells. Bottom panels (C & D) similarly show cross-linked α5β1 integrins also internalize equally well in both cell types. Areas of high integrin internalization localized to increased F-actin staining, indicating F-actin involvement in the endocytosis pathway. α4β1 and α5β1 integrins, red; F-actin, green. Bars, 10μm. Arrows indicate points of integrin internalization.

methods, followed by the tertiary antiserum donkey anti-goat Ab conjugated to Cy5 for 45 min at 4°C to label the proportion of integrins remaining on the plasma membrane. Performing quantitation as indicated in the materials and methods (Section 2.5) we determined that CD81 expression did not affect the amount of α4β1 or α5β1 integrin internalization (Figure 27). Therefore, expression of the tetraspanin CD81 does not influence the uptake of integrins into lipid rafts in monocytic cells.

Section 4.4 Ectopic CD81 expression diminishes integrin capping

While observing the internalization pattern of cross-linked integrins in numerous cells we observed that ectopic expression of CD81 appeared to limit integrin capping. When cross-linking either α4β1 or α5β1 integrin at 37°C in U937 cells we found that in approximately one quarter of the cells the integrins aggregated into caps which occupied less than 50% of the cell volume. However in CD81 transfected cells, the percentage of cells containing integrin caps in less than 50% of the cell volume fell to between 5 and 10% (Figure 28). Therefore, in the CD81 transfected cells, the integrins were more diffusely located within the cell and did not undergo capping as extensively as in the U937 cells. These initial observations suggested that CD81 might limit polarized movement of integrins on the cell surface, but were not compelling.

However, we discovered that when cells were pulsed with nocodazole, an inhibitor of microtubule polymerization, followed by its wash out, we obtained a much more

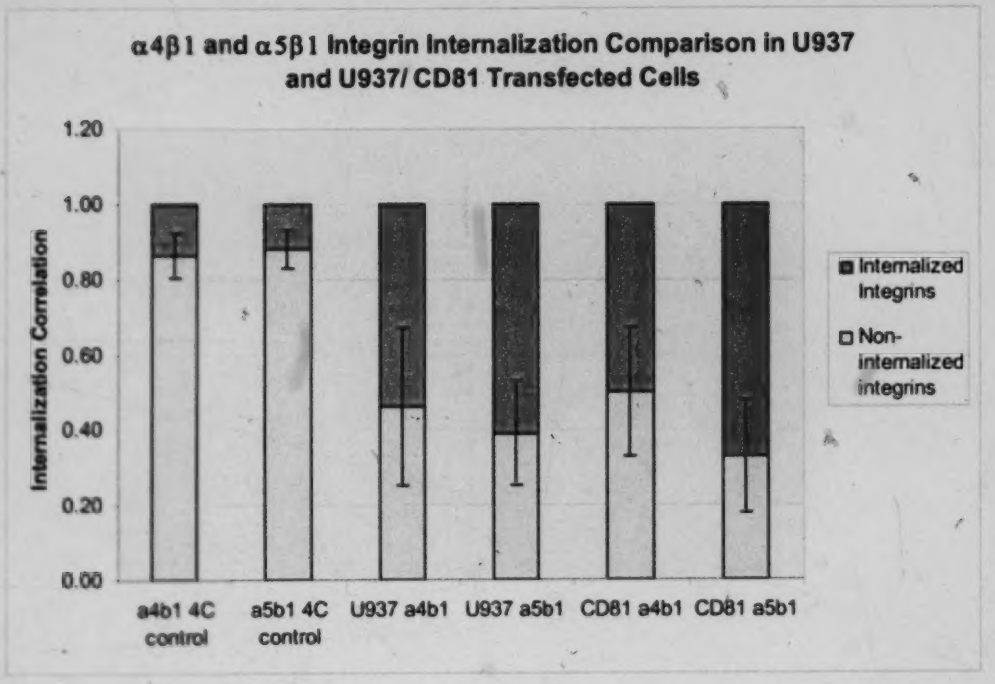

Figure 27. Quantitation of α4β1 and α5β1 integrin endocytosis in U937 and U937CD81 cells. Quantitation was completed using a tertiary antibody system. We first incubated the cells with anti-α4β1 mAb 44H.6 or anti-α5β1 mAb P1D6 at 4˚C for 45min, then cross-linked with goat anti-mouse Ab-Alexa 546 at 37˚C for 60 min. Cells were then incubated with tertiary Ab donkey anti-goat Cy5 for 45 min at 4˚C. Internalization quantitation was completed by taking Z-stack optical sections of 20 individual cells for each sample set. Using Imaris co-localization software, we determined co-localization channel correlations for the data set volume of all 20 cells in each data set. The correlations were then averaged and graphed. Error bars represent +/- 1 standard deviation about the mean. CD81 ectopic expression in U937 cells does not effect the amount of either α4β1 or α5β1 integrin internalization.

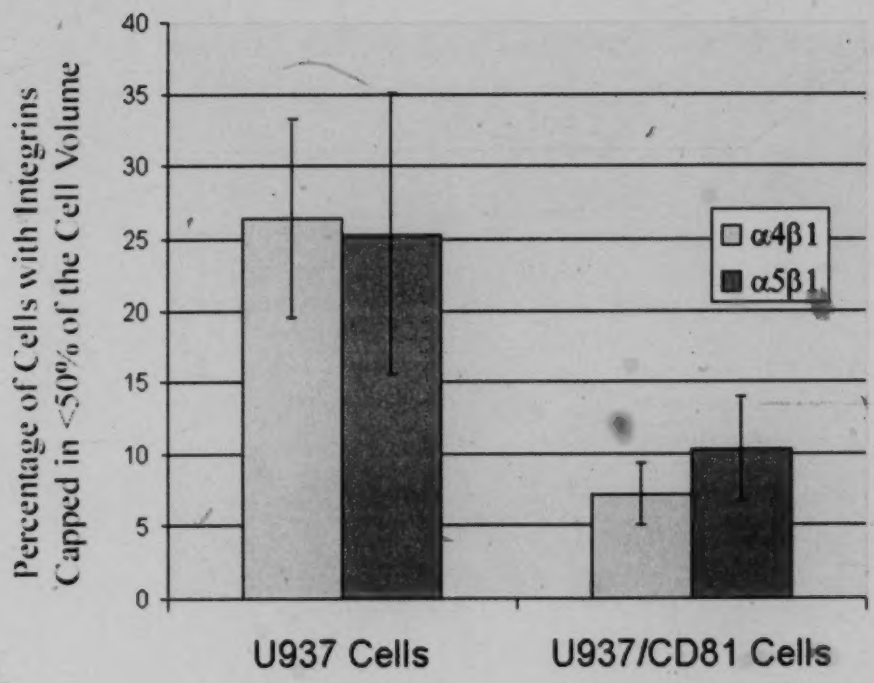

Figure 28. Ectopic expression of CD81 prevents integrin capping upon cross-linking at 37°C. Cells were incubated with anti-α4β1 mAb 44H.6 or anti-α5β1 mAb P1D6 at 4°C, then cross-linked with goat anti-mouse Ab-Alexa 546 at 37°C. Cells were placed on poly-L-lysine and fixed. Single optical sections of over 100 cells per sample were taken at mid-height using confocal microscopy. Cells with all integrins located within an area that was less than 50% of the total cell volume were considered to have large integrin caps. This cell number was compared against the total number of cells to determine the percentage of cells with large integrin caps. As the graph shows CD81 ectopic expression prevent some α4β1 and α5β1 integrin capping. Error bars represent +/- 1 standard deviation about the mean.

dramatic result. When U937 cells were treated with nocodazole for 60 min at 37°C, washed, and integrins were cross-linked with a secondary antibody at 37°C, α4β1 and α5β1 integrins formed large internal integrin caps in proximity to regions of cortical F-actin (Figure 29A and 29C). Contrarily, integrins in the CD81 transfected cells did not form large caps but clustered into much smaller vesicles which underwent internalization (Figure 29B and 29D).

We had already provided evidence that integrins undergo endocytosis in a lipid-raft dependent manner in U937 cells by labeling GM1. We therefore asked whether CD81 would similarly affect the location and mode of internalization of GM1 in U937 cells following treatment with nocodazole and subsequent wash out. We treated cells with nocodazole, washed them and incubated with CtxB-Alexa 594 at 4°C for 45 min, before cross-linking with rabbit anti-CtxB mAb at 37°C for 60 min. Interestingly, we observed that GM1 formed a larger lipid cap in U937 cells but remained distributed in smaller patches in the CD81 transfected cells. Therefore, CD81 antagonizes the movement of clustered integrins and lipid rafts into polarized caps on the cell surface but does not hinder the endocytic process per-se, an effect that is only evident under conditions of temporary disruption of the microtubule cytoskeleton.

We then proceeded to examine whether nocodazole washout treatment would have an effect on the amount of integrin and GM1 internalization in both cell lines. Again, using the tertiary antibody method for quantitation, we first treated the cells with nocodazole at 37°C followed by washing. After removing nocodazole, we cross-linked both integrins

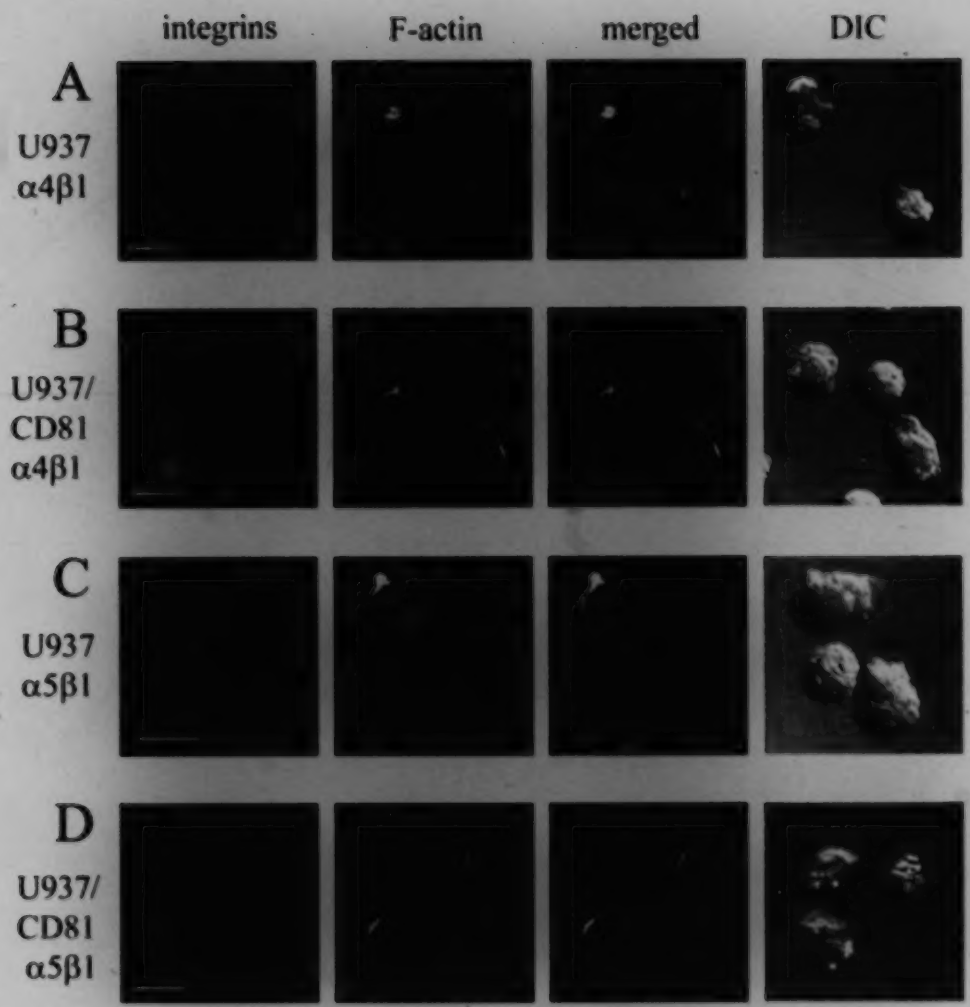

Figure 29. Nocodazole washout treatment causes cross-linked α4β1 and α5β1 integrins to form large aggregates in U937 cells, but not in U937/CD81 cells. Cells were first treated with nocodazole and then washed. Cells were incubated with anti-α4β1 mAb 44H.6 (A & B) or anti-α5β1 mAb P1D6 (C & D) at 4°C, and then cross-linked with goat anti-mouse Ab-Alexa 546 at 37°C. Cells were placed on poly-L-lysine, fixed, permeabilized and stained with phalloidin-FITC to label F-actin. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panels (A & C) show cross-linked α4β1 and α5β1 integrins form large internal integrin aggregates in U937 cells. Integrin α4β1 caps co-localize well with F-actin indicating F-actin involvement in integrin cap formation. Panels (B & D) show cross-linked α4β1 and α5β1 integrins patch and internalize in U937/CD81 cells, indicating CD81 prevents integrin cap formation. α4β1 and α5β1 integrins, red; F-actin, green. Bars, 10μm

and GM1 with secondary antibodies at 37°C and labeled the remaining GM1 and integrins on the cell membrane with tertiary Cy5 antibodies at 4°C. Figure 30 is a representative illustration of α4β1 integrin internalization labeled with tertiary antibody for quantitation. Cross-linked α4β1 integrin clusters and internalizes equally well in both untreated U937 and U937/CD81 cells (Figure 30A and 30B) and some integrin internalization is still observed in cells treated with nocodazole washout (Figure 30C and 30D). Thus it appears that temporary disruption of microtubule polymerization alters the location of integrins, but does not inhibit integrin internalization.

Quantitation of internalization was performed by taking Z-stack optical sections of 20 individual cells for each sample set. Using Imaris co-localization software, we determined co-localization channel correlations for each data set. The correlations were then averaged and graphed. Figure 31 illustrates that CD81 ectopic expression does not alter the amount of integrin or GM1 internalization. As well, nocodazole washout treatment has no effect on the amount of GM1 and integrin endocytosis in U937 monocytic cells. In the CD81 transfected cells, nocodazole washout treatment has a minimal inhibitory effect on integrin internalization but no effect on GM1 internalization. Microtubules antagonize the assembly and modulate the formation of actin stress fibers [90]. Therefore, there appears to be an antagonistic relationship between tubulin polymerization and F-actin. In U937 cells, we found that disrupting this relationship has little to no effect on the amount of integrin and GM1 internalization but does influence the capping capability of the integrins and GM1.

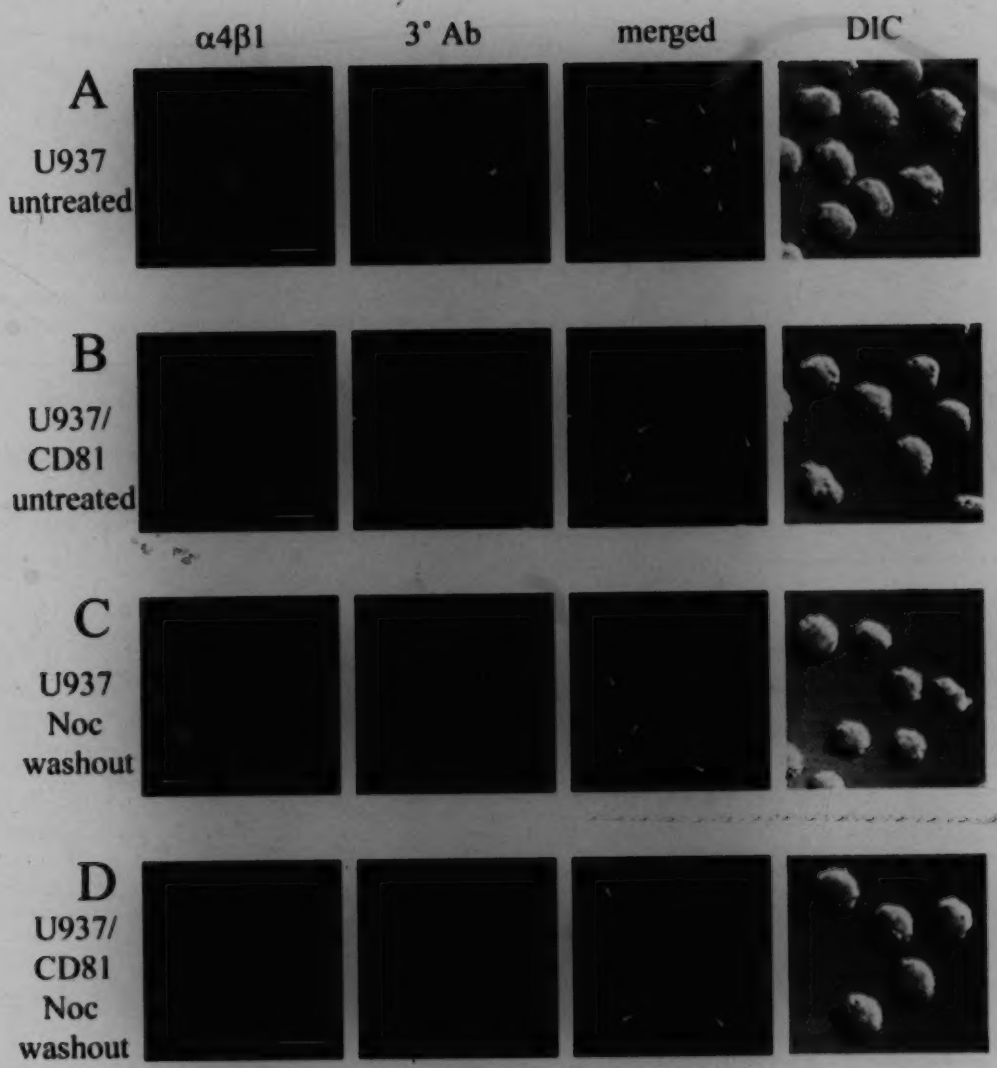

Figure 30. Quantitation of integrin α4β1 internalization in nocodazole washout treated U937 cells and U937/CD81 cells. Cells were first treated with nocodazole and then washed (C & D). Cells were incubated with anti-α4β1 mAb 44H.6 at 4°C, and then cross-linked with goat anti-mouse Ab-Alexa 546 at 37°C. Cells were then incubated with tertiary Ab donkey anti-goat Cy5 at 4°C. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Top panels (A & B) show α4β1 upon cross-linking at 37°C clusters and internalizes well in both cell types with very little co-localization to the tertiary antibody on the plasma membrane. Bottom panels (C & D) show some α4β1 clusters do not co-localize with the tertiary antibody indicating that α4β1 still internalizes despite nocodazole washout treatment. α4β1 integrin, red; tertiary Ab, green. Bars, 10μm. Arrows indicate points of integrin internalization.

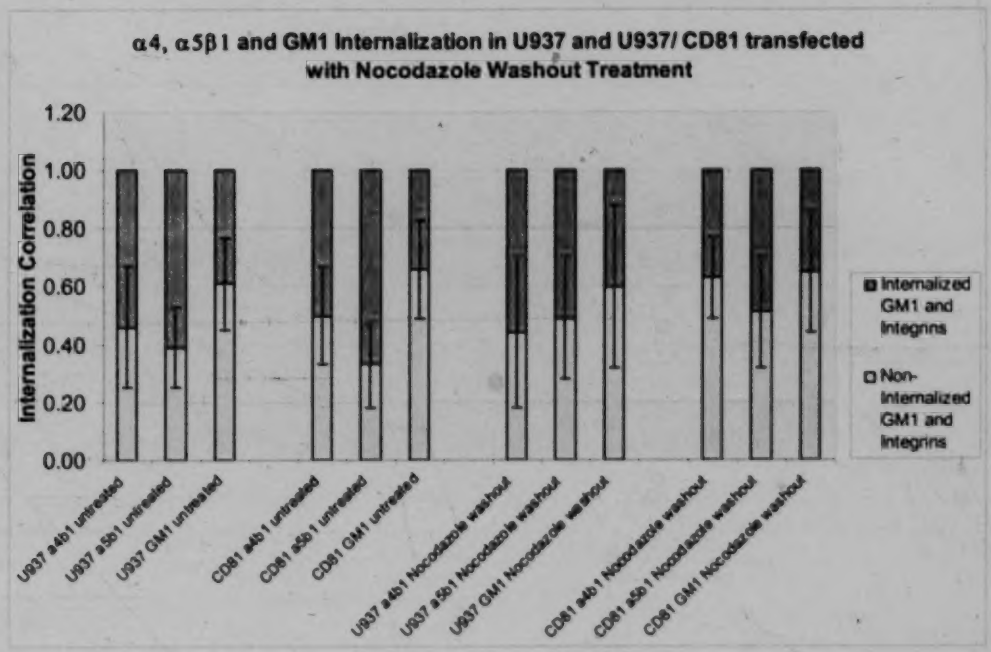

Figure 31. Quantitation of GM1, α4β1 and α5β1 integrin endocytosis in U937 and U937/CD81 cells, with our without nocodazole washout treatment. Quantitation was completed using a tertiary antibody system. Cells were first treated with nocodazole for 60 min at 37°C and then washed. Cells were then incubated with CtxB-Alexa 594 (GM1), anti-α4β1 mAb 44H.6 or anti-α5β1 mAb P1D6 at 4°C for 45min, and then cross-linked with rabbit anti-CtxB or goat anti-mouse Ab-Alexa 546 at 37°C for 60 min. Cells were then incubated with tertiary Ab goat anti-rabbit Cy5 or donkey anti-goat Cy5 for 45 min at 4°C. Internalization quantitation was completed by taking Z-stack optical sections of 20 individual cells for each sample set. Using Imaris co-localization software, we determined co-localization channel correlations for the data set volume of all 20 cells in each data set. The correlations were then averaged and graphed. Error bars represent +/- 1 standard deviation about the mean.
The graph illustrates that CD81 ectopic expression does not affect integrin or GM1 internalization. As well, nocodazole washout treatment has no inhibitory effects on GM1 and integrin endocytosis in U937 and CD81 transfected cells.

Section 4.5 Cytochalasin D treatment abrogates the effects of nocodazole washout treatment

Capping of cell membrane proteins is mediated largely through re-organization of the actin-cortical cytoskeleton [91]. We wanted to know whether actin polymerization is responsible for the exaggerated polar distribution of integrins in U937 cells treated with nocodazole and washed out. We therefore additionally treated cells with cytochalasin D, an inhibitor of F-actin formation. Again, cells were first treated with nocodazole, washed and then exposed to cytochalasin D. Integrins were cross-linked with a secondary antibody at 37°C, and stained with phalloidin-FITC to label F-actin. Interestingly, we found that when U937 cells were treated with nocodazole, washed and exposed to cytochalasin D, cross-linked α4β1 and α5β1 integrins instead of forming large internal integrin caps and F-actin caps, patched into smaller clusters which together with F-actin were diffusely distributed over the plasma membrane (Figure 32A and 32C). Therefore, cytochalasin D treatment abrogates the effects of nocodazole washout indicating F-actin is required for integrin cap formation. In the CD81 tranfectants, cross-linked α4β1 and α5β1 integrins patched and internalized in a similar manner to untreated cells (Figure 32B and 32D). Therefore CD81 appears to oppose actin-dependent capping of integrin-raft complexes and permit local internalization of cross-linked integrins.

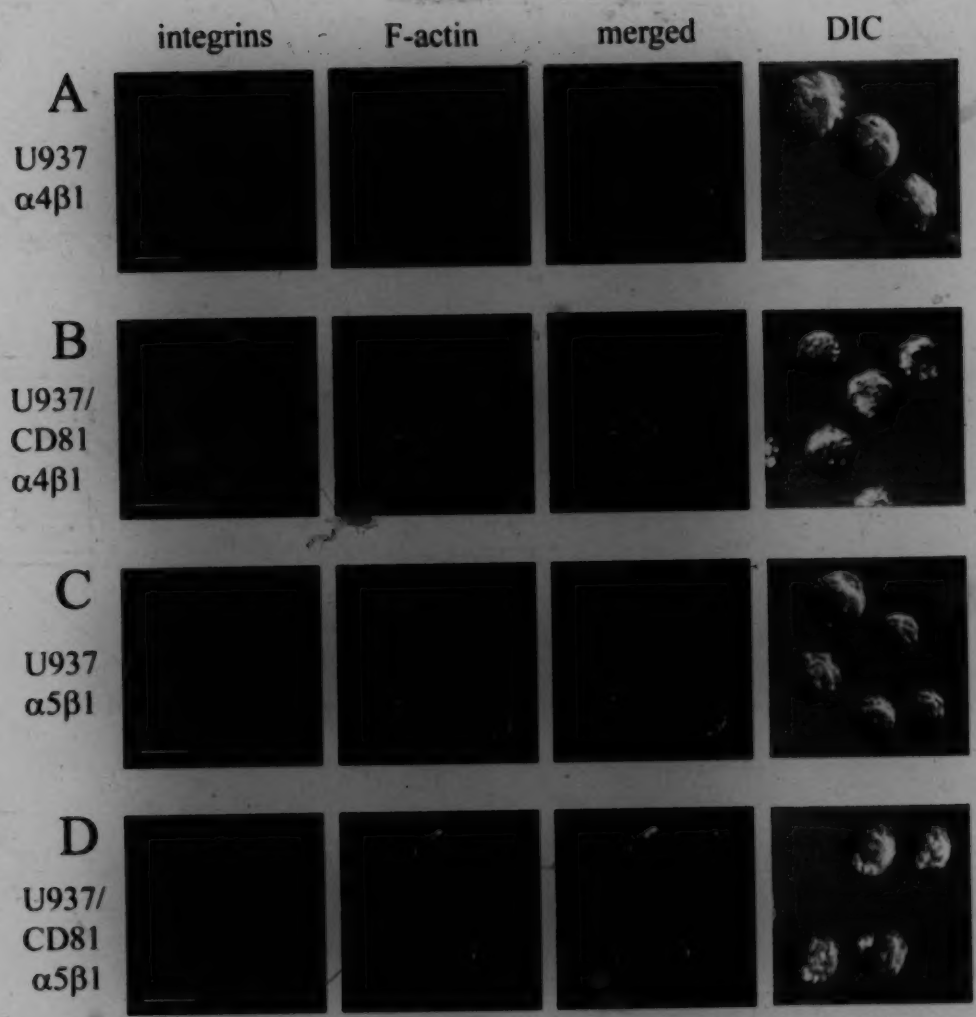

Figure 32. Effect of nocodazole washout treatment on internalization of integrins in U937 cells is abrogated by cytochalasin D treatment. Cells were first treated with nocodazole and then washed. Cells were then treated with cytochalasin D and washed. Cells were then incubated with anti-α4β1 mAb 44H.6 (A & B) or anti-α5β1 mAb P1D6 (C & D) at 4°C, then cross-linked with goat anti-mouse Ab-Alexa 546 at 37°C. Cells were placed on poly-L-lysine, fixed, permeabilized and stained with phalloidin- FITC to label F-actin. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panels (A & C) show cross-linked α4β1 and α5β1 integrins cease forming large internal integrin aggregates upon both nocodazole washout and cytochalasin D treatment and F-actin diffusely distributes on the plasma membrane in U937 cells. Therefore, cytochalasin D treatment reverses the effects of nocodazole washout treatment. Panels (B & D) show cross-linked α4β1 and α5β1 integrins patch and internalize in U937/CD81 cells, despite both nocodazole washout and cytochalasin D treatment. α4β1 and α5β1 integrins, red; F-actin, green. Bars, 10μm

Section 4.6 Trichostatin A treatment abrogates the effects of nocodazole washout treatment

Microtubules provide cells with a structural framework that can be stabilized by post-translational modification. Acetylation of Lys40 of α-tubulin renders microtubules more stable than deacetylated microtubules and less sensitive to depolymerization by nocodazole treatment [92]. A member of the histone deacetylase family, HDAC6, is localized in the cytoplasm and associates with microtubules. HDAC6 functions as a tubulin deacetylase and by deacetylating α-tubulin decreases microtubule stability, and promotes cell movement [93]. Trichostatin A (Tricho A) selectively inhibits HDAC6 and increases the levels of acetylated tubulin, stabilizing microtubules [93]. Therefore, we asked if Tricho A treatment would inhibit the amount of integrin internalization in either U937 or U937/CD81 transfected cells. We treated the cells with 100 nM Tricho A for 24hrs at 37°C, washed and cross-linked the integrins at 37°C as described in the materials and methods. We observed no effect of the Tricho A treatment on the ability of integrins to internalize in both cell sublines. This indicates that increased microtubule stability does not affect the ability of lipid rafts to undergo endocytosis if stimulated.

Since ectopic expression of CD81 appears to affect capping, under conditions of nocodazole washout treatment, when microtubule stability is compromised, we wanted to determine if Tricho A treatment would have an affect on integrin capping in both cell sublines under conditions of nocodazole washout. Cells were therefore treated with 100 nM Tricho A for 24 hrs at 37°C followed by washing. Cells were then treated with 12.5 μg/ml nocodazole at 37°C for 60 min and washed. Both integrins α4β1 and α5β1 and

GM1 were then cross-linked with secondary antibodies at 37°C as described in the materials and methods. Again, the membrane was stained with fast DiO. As seen previously, nocodazole washout treatment by itself produced large internal integrin caps in U937 cells (Figure 33A) whose formation were inhibited by CD81 expression (Figure 33C). Interestingly, upon additional Tricho A treatment, we see complete abrogation of the nocodazole washout effect in the U937 cells. The U937 cells ceased forming large internal integrin aggregates, and the integrins clustered into smaller patches, whereas in the CD81 transfected cells, Tricho A treatment had no effect on integrin internalization. We observed the same results when α5β1 or GM1 was cross-linked. Since additional Tricho A treatment in U937 cells results in the same phenotype as the CD81 transfected cells, a nice speculation would be that CD81 ectopic expression may be influencing the stability of microtubules by modulating HDAC6 activity. To investigate these ideas, we attempted to show an increase in microtubule stability by staining for α-tubulin with anti-α-tubulin mAb (Sigma). Unfortunately, in our U937 monocytic cells we were unable to achieve clear resolution of microtubule structures. Consequently, despite our interesting results, we were unable to confirm that Tricho A treatment inhibited α-tubulin acetylation.

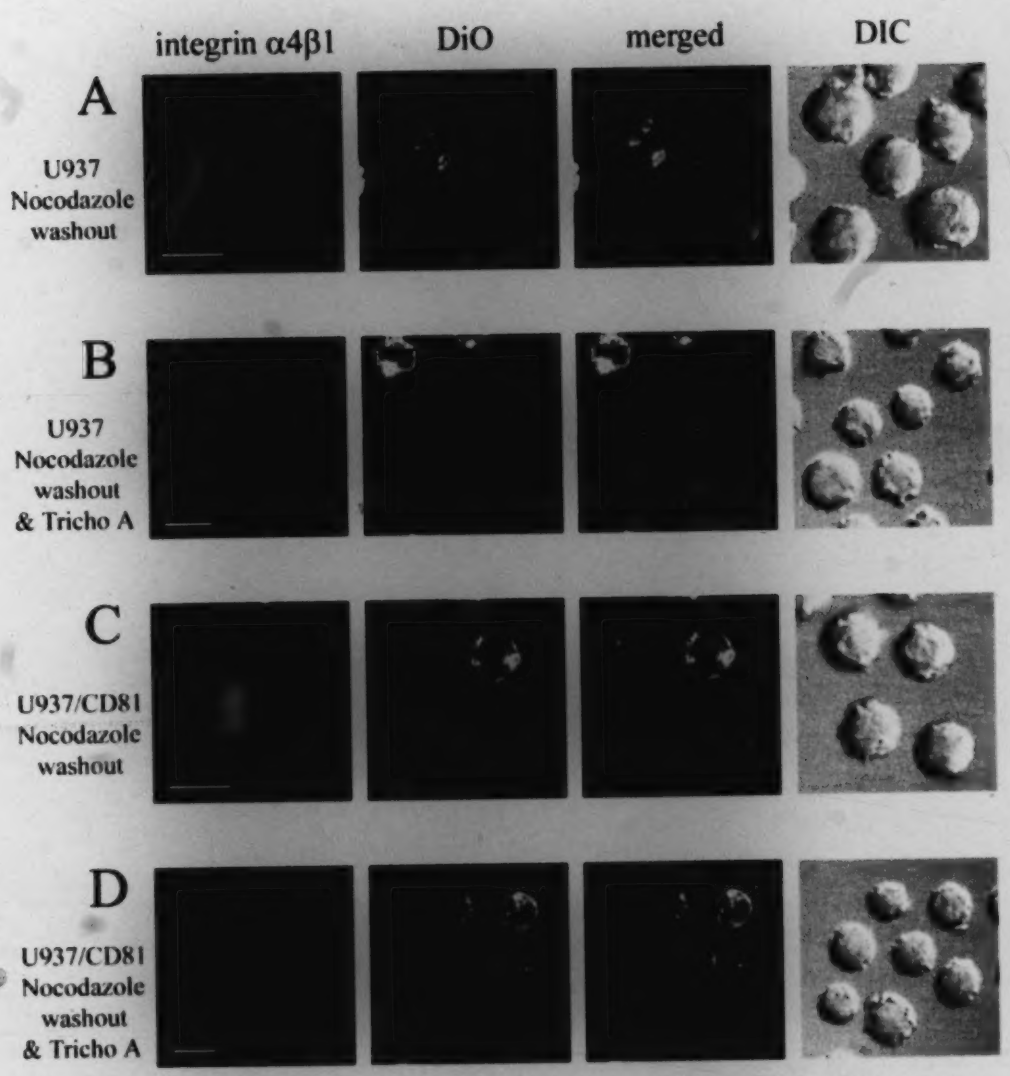

Figure 33. Effect of nocodazole washout treatment on internalization of integrins in U937 cells is abrogated by trichostatin A (Tricho A) treatment. Cells were first treated with Tricho A for 24 hrs and then washed. Cells were then treated with nocodazole at 37°C, washed, incubated with anti-α4β1 mAb 44H.6 at 4°C, and then cross-linked with goat anti-mouse Ab-Alexa 546 at 37°C. Cells were placed on poly-L-lysine and fixed. Single optical sections of cells shown were taken at mid-height using confocal microscopy. Panels A and C show cross-linked α4β1 integrin in U937 (A) and U937/CD81 cells (C) with nocodazole washout treatment. In the U937 cells, α4β1 forms large integrin aggregates (A), which is inhibited by CD81 expression in the transfected cells (C). Panels B and D show cells treated with both nocodazole washout and Tricho A. The U937 cells, upon additional Tricho A treatment, cease forming large internal integrin aggregates, whereas Tricho A treatment has no effect on the CD81 transfected cells. α4β1 integrin, red; DiO, green. Bars 10μm.

Section 4.7 Chapter summary

Ectopic expression of CD81 did not influence the amount of internalization of α4β1 or α5β1 integrin. However, when the organization of the microtubular cytoskeleton system was disrupted by nocodazole exposure and subsequent washout, CD81 expression dramatically affected the route of integrin internalization. In U937 cells, the integrins aggregated into polarized caps containing F-actin and endocytosed as a few large caps. However, in CD81 transfectants the clustered integrins formed small patches which internalized locally. CD81 expression had exactly the same effect on the internalization of GM1 as on the internalization of α4β1 integrin. When cells were simultaneously treated with cytochalasin-D the polarizing effect of nocodazole washout in U937 was abrogated, indicating that polymerization of cortical actin drives the assembly of integrin caps. Since CD81 modulates dynamic interactions between the two interactive cytoskeletal systems, microtubules and F-actin, and associates with lipid rafts, we propose that the role of CD81 may be to modulate the interaction between lipid raft-associated integrins and the cytoskeleton. Similarly, when U937 cells were simultaneously treated with nocodazole washout and Tricho A, integrin capping was abolished indicating that increased microtubule stability counteracts the nocodazole depolymerizing effect on microtubules. Since increased microtubule acetylation, abrogates the nocodazole washout effect, and CD81 expression also abrogates the nocodazole washout effect, an interesting possibility is that CD81 may regulate the acetylation of microtubules through HDAC6.

Chapter 5: Discussion

Section 5.1 Lipid rafts mediate β1 integrin endocytosis in monocytes

The key finding of my thesis is that clustering either α4β1 or α5β1 integrins on monocytic cells leads to the formation of macroscopic patches containing the ganglioside GM1 which internalize through the activation of a lipid raft-dependent endocytic pathway. Ligating α4β1 integrin with a directly conjugated fluorescent primary mAb did not induce internalization. To induce internalization both primary and secondary antibodies are required indicating that triggering of endocytosis requires supramolecular clustering of the integrin. The ability of antibodies to promote internalization of integrins has a precedent in studies demonstrating that cross-linking the integrins α6β1 and α6β4 with multivalent antibodies induces internalization of the integrins in epithelial cells and keratinocytes [17], and that α5β1, but not α4β1 internalizes following cross-linking in several lymphoid cell lines [16]. However, the mechanisms of internalization in these studies were not investigated. The internalization of α5β1 but not α4β1 integrins in the U937 cell line [16] conflicts with our findings. This discrepancy could reflect their use of the mAb HP2/4 to ligate α4β1 integrin whereas we used mAb 44H.6. Integrins ligated with different mAb have been observed to exhibit significantly different rates of endocytosis [17]. Since antibodies bind to different epitopes they may support

different conformations of the molecule or differentially block sites of interaction with other proteins. Whether mAb 44H.6 promotes association of the α4β1 integrin with lipid rafts whereas mAb HP2/4 does not is therefore an interesting question for future study.

Recent evidence, demonstrating that integrins gain adhesive functionality through association with GM1-containing lipid rafts [19] and that integrins assemble lipid rafts at points of adhesive contact [8], indicate that lipid rafts play an essential role in bi-directional communication between the cytoskeleton and the ECM. Additionally, cross-linking α6β4 integrin with antibodies promoted both aggregation of the integrin and its redistribution into lipid rafts, a process which was required for integrin-mediated signaling [64]. Previous studies in U937 cells have also indicated the importance of lipid raft-dependent internalization and trafficking of the CD4 antigen into intracellular degradative compartments [94]. Therefore, we propose that association of α4β1 and α5β1 integrins with lipid rafts is a prerequisite for integrin internalization in monocytic cells. In support, lipid rafts in caveolin-1 null cells invaginate to form caveolae-like membrane pits which internalize so rapidly that they are only visible when budding is inhibited. This suggests that the endocytic potential of lipid rafts is significant and that caveolin-1 supports long-term expression of caveolae to aid in cargo selection [41]. To determine if integrin endocytosis is indeed lipid raft-mediated in U937 cells, we marked the lipid rafts by labeling GM1, a commonly used lipid raft marker [19, 45, 95], and cross-linked the integrins. Since we observed that many internalized integrins co-localized with GM1 we conclude that integrins internalize in vesicles enriched in lipid rafts.

However, caveolin-1 was shown to contribute to integrin clustering and co-precipitated with integrins, suggesting that integrins associate with caveolae to signal [96]. Very recently, it was demonstrated that integrins not only signal but can internalize through association with caveolae. Interestingly, in this study the primary interaction of α2β1 integrin was with lipid rafts which provided a vehicle for transport of the clustered integrin along actin filaments to enter caveolae where they were packaged into endocytic vesicles [97]. This indicates that integrins associate with lipid rafts and that caveolae serve as regulatory domains for the assembly of endocytic vesicles. A caveolin-dependent mechanism is unlikely to support endocytosis in our experiments since blood monocytes fail to express, or express very low levels of caveolin-1 [42] and using RT-PCR analysis and confocal microscopy we confirmed that the U937 monocytic cell line is deficient in caveolin-1, and therefore lacks caveolae [98]. Our data support the concept that lipid rafts are the primary mediators of integrin trafficking while demonstrating that lipid rafts contain the machinery to mediate endocytosis. Our study therefore demonstrates for the first time that lipid rafts can mediate integrin endocytosis independently of caveolae and provides support for the emerging concept that caveolae serve as specialized domains to focus and regulate an endocytic process where lipid rafts are the engine of the lipid-mediated endocytosis

To further support our claim that integrins expressed on monocytes internalize via lipid rafts we treated the cells with MβCD. Because MβCD effectively depletes cholesterol within the external leaflet of the plasma membrane it is commonly used as a tool to disrupt lipid rafts [7]. We found that following brief exposure to 15 mM MβCD,

integrins lost their punctuate appearance on the plasma membrane and became diffusely distributed. At the same time integrin endocytosis was completely abolished indicating that lipid rafts play an important role in both clustering integrins into larger assemblies and in mediating their internalization. MβCD treatment, especially at higher concentrations and over long periods of application, can compromise the integrity of the plasma membrane [99]. However, it is generally agreed that short exposures to MβCD differentially affect lipid raft function and have little effect on other cellular processes [100]. In our experiments we incubated cells with MβCD for just 15 minutes. Furthermore, although MβCD treatment inhibited integrin internalization, it did not affect adhesion to ECM under static conditions indicating that cellular function is not compromised under the conditions of our experiment. Many processes involving vesicle trafficking, including clathrin-mediated endocytosis, are affected to some extent by cholesterol-depletion. Since lipid rafts are particularly sensitive to cholesterol-depletion the ability of MβCD to completely inhibit integrin-mediated endocytosis argues that MβCD is affecting integrin endocytosis through the disruption of lipid rafts.

Integrin internalization was first described to be consistent with clathrin-mediated endocytosis [18] and despite our evidence that integrin internalization is dependent on lipid raft integrity we needed to exclude clathrin-coated pits as a possible mediator of integrin endocytosis. On the plasma membrane, clathrin forms clathrin-coated membrane invaginations that recruit cell-surface receptors for internalization. The clathrin-coated pit then pinches off to form an endocytic vesicle [36]. However, through development of dominant-negative clathrin mutants, it was realized that some proteins

also utilize non-clathrin-mediated endocytic pathways [36]. In fact, lipid raft markers have been shown to undergo endocytosis independently of clathrin-coated pits and may actually be prevented from entering clathrin pits [95]. To exclude the possibility that clathrin-coated pits mediated integrin endocytosis we treated cells with a high molar concentration of sucrose (0.45M), a commonly used method [101, 102], for disrupting clathrin-dependent endocytosis [81]. As a control, we labeled the cells with directly-transferrin, which endocytoses in a clathrin-dependent pathway. We observed that hypertonic sucrose solution did indeed inhibit internalization of transferrin but did not abolish endocytosis of cross-linked integrin or GM1 indicating these endocytic pathways are clathrin-independent.

In addition to demonstrating that α4β1 and α5β1 integrins internalize in a lipid raft-mediated pathway we show that integrins preferentially associate with clustered lipid rafts in monocytes. We observed that clustering GM1 was sufficient to pull integrins into GM1-enriched assemblies from which GM1 internalized, leaving the integrins behind on the plasma membrane. This suggests integrins have an affinity for lipid rafts but require engagement and clustering in order to internalize. This implies the existence of mechanisms which differentiate between occupied and non-occupied integrins. Such mechanisms are probably essential to conserve unoccupied receptors on the cell surface. How that discrimination is effected is an interesting question and implies the existence of sorting mechanisms that admit lipid rafts to endocytic vesicles while excluding unoccupied integrins. Sorting might be effected at the neck of the forming endosome where membrane curvature imposes limitations on the diffusion of transmembrane

proteins. It can be envisioned that only those molecules with a high affinity for lipid rafts would be able to diffuse past the bottleneck. According to this model the affinity of the integrin for the lipid raft would determine inclusion in the endosome. Antibody engagement could increase the affinity of integrins for lipid rafts through multimerization and through stabilization of open conformations associated with ligand binding. However, other mechanisms may also play a role. Integrins undergo reversible associations with the actin cytoskeleton which limit their diffusion in the plane of the membrane [103]. Such interactions may impede recruitment of unoccupied integrins into endosomes. Integrin occupation signals which abrogate those interactions may therefore be required to facilitate inclusion. In any case, our findings demonstrate that integrins have a constitutive and selective affinity for lipid rafts compared to MHC class I and transferrin which do not co-localize with GM1.

The ability to promote endocytosis simply by clustering GM1 supports the concept that lipid rafts in promonocytic cells contain machinery for endocytosis which can be triggered by integrin-mediated clustering of the raft. The potential of lipid rafts to internalize extends to other lineages since ligating GM1 with CtxB stimulates endocytosis in T lymphocytes [47]. In common with β1 integrin-triggered endocytosis, GM1 internalization was sensitive to inhibition by MβCD treatment indicating a requirement for cholesterol. This concurs with studies in caveolin-1 deficient cells, demonstrating that cholesterol depletion does not affect the ability of cholera toxin to bind GM1 but effectively prevents the complex from entering the cell [104]. This indicates that lipid rafts are not required to mediate receptor recognition of ligand, but

rather to provide the necessary membrane organization. Lipid rafts may serve to sort cargo, or to recruit signaling molecules and cytoskeletal proteins required to stimulate vesicle formation, budding and endocytic trafficking.

Monocytes, as components of the immune system, contain FcγRI and FcγRII Fc receptors that undergo internalization upon immunoglobulin binding [85]. Utilizing the biotin/streptavidin complex to cross-link GM1 and antibody fragments devoid of Fc regions to cross-link the integrins allowed us to cluster our target surface molecules without activating Fc receptor-mediated endocytosis. We demonstrated that GM1 and integrins internalize in Fc receptor-independent pathway, further supporting that GM1 and the integrins internalize in a lipid raft-dependent and Fc-independent pathway.

Our finding that lipid raft-mediated integrin endocytosis is a property of U937 and THP-1 monocytic cells, but not of promyelocytic or B cells, demonstrates that lipid rafts perform different functions in different cells. If lipid rafts determine the function of a cell type then it might be anticipated that as cells acquire more sophisticated functions they sacrifice some of the more basic functions. In this regard, pro-monocytes with their limited signaling and phagocytic capacity might represent a default state in which integrin ligation simply means "clear me". U937 is an acute myelogenous leukemia histiocytic cell line but is considered a pro-monocyte because U937 cells express markers and contain specific properties of monocytes [105]. THP-1 is also a leukemia cell line that expresses distinct monocytic markers [106] and is considered to be very similar to the U937 cell line. However, with differentiated function more complex

associations between raft components may require more sophisticated responses to integrin ligation involving chemotactic gradients or phagocytosis. Mono Mac-6 is a mature monocytic cell line which constitutively exhibits phagocytosis and expresses markers of mature monocytes absent from U937 and THP-1 cells [107]. Mono Mac 6 cells may therefore utilize lipid rafts in specialized ways such as chemotaxis and phagocytosis.

Interestingly, we observed that clustered integrin, but not clustered GM1, internalizes in HL-60 cells and that it did so in a lipid raft-independent pathway. HL-60 is a promyelocyte, a precursor granulocyte of the neutrophil lineage [108]. Neutrophils migrating on fibronectin recycle β1 integrins from the lagging end to the leading edge in endocytic recycling compartments through clathrin-dependent endocytosis [65]. Our observation that clustering α4β1 induces internalization whereas GM1 does not supports the importance of clathrin-dependent recycling of β1 integrins in cells of the neutrophil lineage for migration which differ from monocytes.

Neither cross-linked integrins nor GM1 internalized in Raji. Raji cells are a B-lymphocyte cell line, distinctly different from monocytes. In B-cells, lipid rafts are implicated in signaling associated with engagement of the B-cell receptor [34]. Therefore, engagement of integrins in B-cells may trigger lipid raft interactions that modulate cognate function rather then internalization. Although these ideas are speculative our results clearly indicate that cells differ markedly in their response to integrin ligation. Since the properties of lipid rafts inevitably reflect the properties of the

proteins that populate them, lipid raft mediated integrin endocytosis may be a default mechanism that is readily displaced by signaling requirements in more differentiated cells.

We have demonstrated that cross-linking either α4β1, α5β1 integrin or GM1 in monocytes induces lipid-raft dependent endocytosis. But what is the functional relevance of rapidly internalizing integrins when engaged with antibody? To understand this phenomenon, we cross-linked fibronectin at 37°C, the common ligand of both α4β1 and α5β1 and observed its rapid internalization in both U937 and THP-1 cells. Fibronectin is a component of the ECM and soluble fibronectin may resemble loosely bound matrix debris. Monocytes actively participate in immune response and traffic to sites of inflammation. The functional significance of endocytosing fibronectin may be that the cell can modify or modulate the extracellular matrix [18] whether for attachment and migration or for degradation of the extracellular matrix required for extravasation.

In contrast to α3β1, which trafficks into the lysosomal degradation pathway [109], β1 integrins in breast epithelial cells traffic within a clathrin-dependent endosomal recycling compartment containing transferrin receptors [110]. However, we found that internalized β1 integrin did not associate with either lysosomes or recycling endosomes. One explanation is that our incubation time of two hours to allow integrin association with transferrin or lysosomes was too short, and that even though the rate of internalization is rapid, further integrin trafficking may be much slower. However, α2β1 which internalizes in a caveolar pathway, also failed to enter classical recycling endosomes [97].

Therefore, a second explanation is that β1 integrins internalize and traffic within a unique pathway in monocytes. One possibility is that fibronectin or antibody trafficks to lysosomes where it is degraded whereas integrins fuse with endosomes and are shipped back to the membrane. This would help monocytes preserve their membrane receptors and prevent cells from using excess energy to synthesize new fibronectin receptors. This trafficking pathway may take longer than two hours, explaining why we do not observe integrin association with the lysosomal or endosomal markers.

To understand the cytoskeletal and signaling requirements of lipid raft-dependent endocytosis we quantitated the rate of integrin and GM1 internalization in cells treated with various inhibitors to either disrupt the cytoskeleton or inhibit downstream signals. We found that lipid raft endocytosis was dependent on F-actin polymerization and serine/threonine kinase activity. Coinciding with our data, lipid raft-mediated endocytosis of GPI anchored proteins, which is also clathrin and caveolin-1 independent, requires both actin reorganization and PKC activation (a serine/threonine kinase) [111]. Numerous studies have indicated PKCα activity as a requirement for integrin endocytosis [97, 110] but U937 cells only express PKC isozymes β1, β2, ε and ζ [112]. Since staurosporine is a broad inhibitor of serine/threonine kinases we could not identify the specific kinase involved in integrin and GM1 endocytosis in monocytes. Future experiments could involve treating U937 cells with specific serine/threonine kinase inhibitors to identify the kinase required. F-actin polymerization was shown to be involved in lipid raft coalescence in T-cells to induce T-cell activation [91] and directly involved in lipid raft invagination during GPI-anchored protein endocytosis [111].

Therefore, F-actin disruption may have prohibited sufficient clustering of lipid rafts and lipid raft invagination to induce endocytosis.

However, we did find some discrepancies between integrin and GM1 endocytosis. We observed that integrin internalization is more sensitive to wortmannin treatment than GM1 internalization. Therefore, integrin endocytosis may additionally require PI-3 kinase activity. PI-3 kinase activity was previously shown to be a requirement for both β1 integrin signaling [113] and trafficking [110]. Integrin endocytosis was only dampened by wortmannin treatment and not abolished and since integrin function, which involves integrin signaling, requires association with lipid rafts, perhaps PI-3 kinase activity heightens the ability of integrins to relocate to lipid rafts.

In conclusion, using monocytic cells lines we have demonstrated that upon cross-linking the α4β1 and α5β1 integrins rapidly internalize in the same vesicles as GM1 in a cholesterol-dependent pathway indicating that integrins internalize via lipid rafts. We also show that cross-linking fibronectin on the cell surface induces fibronectin internalization supporting a role for lipid raft-mediated endocytosis in clearance of ligand-occupied receptors from the cell surface through a cytoskeleton and serine/threonine kinase dependent pathway. This study supports the views that lipid rafts can provide an alternative means to clathrin for internalization [41]. The benefit of such a pathway to pro-monocytes may be to maximize the effectiveness of integrin adhesion for motility and extravasation. However, such a mechanism might be harnessed by monocytes to assist in the scavenging and degradation of ECM in areas of wounding and

tissue reorganization.

Section 5.2 CD81 regulation of integrin lipid raft-mediated endocytosis

We have demonstrated that the two major integrins, α4β1 and α5β1, in U937 cells undergo lipid raft-mediated endocytosis. U937 cells lack expression of the tetraspanin CD81, which modulates integrin function and associates with both lipid rafts and integrins. Since tetraspanins act as adaptor proteins, we hypothesized that CD81 would aid in the redistribution of integrins into lipid rafts where they could associate with other signaling proteins and influence integrin endocytosis. We tested our hypothesis by transfecting CD81 into U937 cells. Our hypothesis is supported by the role of tetraspanins in regulating β1 integrin recycling [114], in a PKC dependent manner [110, 114]. Tetraspanins are thought to contribute to β1 integrin recycling by acting as linker proteins bringing integrins into close proximity with PKC, with subsequent phosphorylation, integrins can associate with effector molecules [115]. However, in this study we proved our hypothesis wrong and demonstrated that CD81 ectopic expression in U937 cells, does not alter the rate of integrin and GM1 uptake upon engagement. This suggests that the integrins in monocytic cells, shuttle into lipid rafts upon engagement and associate with signaling proteins such as PKC equally well with or without the presence of CD81 ectopic expression.

Despite this setback, upon close observation, we noticed that in many cells, clustered integrins formed super-clusters which formed caps before internalizing. Ectopic expression of CD81 diminished integrin capping formation. These results are interesting because they suggest that CD81 is influencing the clustering as opposed to the endocytic capability of the integrins. Microtubules and F-actin are involved in an antagonistic relationship with each other and microtubule breakdown can stimulate actin formation [90]. To disrupt the relationship between the microtubule and actin cytoskeleton we treated the cells with nocodazole which inhibits microtubule polymerization, and then washed nocodazole from the cells. Since nocodazole treatment is reversible removing it allows microtubules to re-polymerize. However, by temporarily preventing microtubule but not actin polymerization the balance between microtubules and actin is disrupted, giving F-actin the opportunity to polymerize without microtubule control. When integrins and GM1 were engaged in nocodazole-pulsed cells we observed dramatic differences between the two cell lines. Whereas integrins and GM1 capped and internalized as large vesicular caps in U937 cells they formed smaller clusters that internalized locally in CD81 transfectants. Therefore, CD81 influences the ability of integrin and GM1 containing lipid rafts to cap.

Interestingly, caps containing $\alpha4\beta1$ co-localized with F-actin, indicating F-actin may play a role in the capping process. The $\alpha4$ subunit is the only integrin α–subunit known to directly bind paxillin, an adaptor protein that associates with F-actin [60]. This may explain why we observe $\alpha4\beta1$ integrin caps associating with F-actin and not with $\alpha5\beta1$ integrin in U937 cells. To further examine the role of F-actin in cap formation, cells

were additionally treated with cytochalasin D, an inhibitor of F-actin polymerization. Cytochalasin D treatment completely abrogated the nocodazole washout effect and diminished cap formation in U937 cells, supporting a role for F-actin in integrin cap formation.

Therefore, CD81 opposes F-actin dependent capping of integrins and lipid rafts under conditions of microtubule disruption. Tetraspanins act as conditional links between integrins and F-actin to elicit integrin function [116]. For instance, CD82, which also associates with $\alpha 4\beta 1$ integrin, [72] interacts with the cytoskeleton and permits both cytoskeletal rearrangement and co-stimulatory integrin activity [52]. This is accomplished by the extracellular region of CD82 which interacts with the α domain of the integrins. CD82, in turn recruits signaling molecules such as PKC into close proximity of the integrins to activate down stream integrin-mediated signals. [117]. Similarly, CD151, also part of the tetraspanin family, modulates cytoskeleton interaction with integrins through its cytoplasmic tail [118]. Therefore, tetraspanins provide topological organization which guides the clustering of different tetraspanin-associating proteins on the cell surface, allowing proper signaling to mediate cytoskeleton reorganization [119]. Tetraspanins have also been shown to link lipid rafts to the actin cytoskeleton [120] therefore, we propose that the role of CD81 may be to modulate the interaction between lipid rafts, integrins and the cytoskeleton. Since CD81 expression alters the route of integrin/GM1 internalization but not the amount of internalization, such interactions regulate actin-mediated lateral migration of integrin-raft complexes within the plane of the plasma membrane, but not their internalization. Given that CD81

opposes migration of these assemblies only in cells which have been treated to temporarily disrupt the balance between microtubule and actin formation, CD81 may assist microtubule antagonism of actin polymerization by enhancing microtubule polymerization. This is supported by pre-treating cells with Tricho A to enhance the acetylation of tubulin which prevented capping of GM1 and integrins in the presence of nocodazole in U937 cells. Tricho A, increases the stability of microtubules by enhanced acetylation through inhibiting the histone deacetylase HDAC6 [93]. Since, ectopic expression of CD81 acts similarly to Tricho A to inhibit capping, CD81 may regulate microtubule acetylation through HDAC6. Inhibiting HDAC6 by Tricho A increases the levels of microtubule acetylation [93]. Therefore, CD81 may negatively regulate HDAC6 or positively regulate an acetylase to increase microtubule stability through acetylation, counteracting the effects of nocodazole washout treatment. Since, microtubule stabilization inhibits F-actin polymerization, and integrin/GM1 capping is F-actin dependent, CD81 expression can decrease F-actin polymerization, by inhibiting HDAC6 and therefore diminish capping which correlates with our observations.

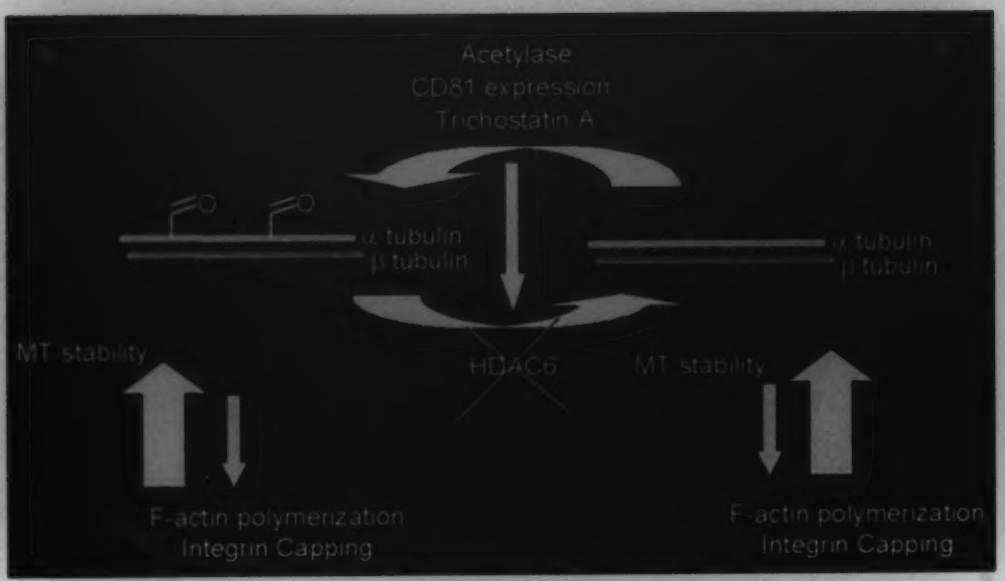

Figure II. Potential functions of CD81. CD81 ectopic expression may regulate microtubule stability by positively regulating histone acyetylases or by negatively regulating HDAC6.

In conclusion, we demonstrate for the first time that cross-linking the two major integrins of pro-monocytic cells, α4β1 and α5β1, results in their rapid internalization through a lipid sorting event. We show that monocyte lipid rafts contain all the necessary machinery to induce endocytosis and that this pathway is dependent on cholesterol, activation of serine/threonine kinase and cytoskeleton reorganization. Therefore, our study supports the view that lipid rafts can mediate endocytosis independently of clathrin or caveolae. This could potentially open a new avenue of cancer treatment in which lipid rafts provide an alternative mechanism for the effective internalization of anti-cancer drugs. We also provide evidence that CD81 modulates the actin cytoskeleton through regulation of microtubule assembly, a novel concept which upon further investigation may help to elucidate the elusive function of the tetraspanin family.

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